Gene therapy with naked DNA: Potential steps towards deregulation

Joost H. van den Berg and John B.A.G. Haanen

Division of Immunology
The Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

This report was commissioned by COGEM. The contents of this publication are the sole responsibility of the authors and may in no way be taken to represent the views of COGEM.

Dit rapport is in opdracht van de Commissie Genetische Modificatie samengesteld. De meningen die in het rapport worden weergegeven zijn die van de auteurs en weerspiegelen niet noodzakelijkerwijs de mening van de COGEM.

Table of Content

1 Samenvatting ...3
2 Summary ...6
3 Introduction ...8
4 Genomic integration in somatic cells ...13
4.1 Effect of novel vectors on genomic integration ...15
4.2 Effect of delivery technique on genomic integration ...16
4.3 Effect of synthetic carriers on genomic integration ...16
5 Genomic integration in germ line cells (vertical transmission) ...18
5.1 Plasmid uptake via the oral route ...18
6 Recombination with viruses ...20
7 Environmental release of DNA ...21
8 Bacterial uptake and persistence (horizontal transmission) ...23
8.1 Novel vectors lacking antibiotic resistance genes ...24
8.2 Effect of novel developments on risk of horizontal transmission ...25
9 Conclusion ...27
10 Advice ...29
References ...30
Appendix I: Environmental risk of DNA containing nanoparticle formulations ...38
Appendix II: Environmental risk of DNA tattoo vaccination ...40

1 Samenvatting

Naakt of plasmide DNA (plasmide DNA toegediend middels een niet-virale methode) wordt frequent gebruikt als vector in gentherapie en DNA vaccinatie toepassingen. Plasmide is een cirkelvormige streng bacteriëel DNA, dat zich buiten het chromosomale DNA bevindt en dat met behulp van recombinant DNA technieken kan worden voorzien van een gen van interesse, het zogenaamde insert. Om goede expressie van dit gen in eukaryotische cellen te krijgen, bevat het plasmide DNA ook nog een aantal andere sequenties, zoals promoter- en polyadenylatiesequenties. Daarnaast is er in het plasmide een "origin of replication", veelal in combinatie met een antibioticum resistentiegen geïncorporeerd. Deze sequenties zijn noodzakelijk voor respectievelijk replicatie en retentie in de productiecel *E.coli.* Het gebruik van naakt DNA brengt een theoretisch milieurisico met zich mee met een kans op het ontstaan en verspreiden van genetisch gemodificeerde organismen (ggo's). Theoretisch kunnen onbedoeld nieuwe ggo's ontstaan door de opname van naakt DNA door somatische cellen, kiembaancellen, virussen of bacteriën. Uit de vorige in 2004 uitgevoerde studie, geïnitieerd door de COGEM, bleek dat het milieurisico van naakt DNA voor het gebruik in mensen (en dieren) minimaal is. Dat rapport adviseerde om voor toepassingen in mensen onderscheid te maken tussen 3 veiligheidsklassen van plasmide DNA's. Deze studie diende als uitgangsonderzoek voor het huidige rapport, dat gebaseerd op voortschrijdend inzicht en onderzoek een nog verdere vereenvoudiging van de regelgeving betreffende het gebruik van plasmide DNA in mensen voorstelt.
De afgelopen jaren is er veel onderzoek gedaan om de relatief lage transfectie-efficiëntie, één van de tekortkomingen van naakt DNA t.o.v. virale vectoren, te verbeteren. In dit onderzoek kan grofweg onderscheid gemaakt worden tussen het ontwerpen van 1.) verbeterde DNA sequenties 2.) synthetische dragers en 3.) nieuwe fysische en mechanische toedieningstechnieken.
In dit rapport zijn de huidige milieurisico's van naakt DNA gereëvalueerd en is gekeken of nieuwe ontwikkelingen in het veld invloed kunnen hebben op dit risico.

Integratie van naakt DNA in het genoom van somatische cellen kan na toediening in theorie plaatsvinden, hetgeen onder extreme omstandigheden ook is aangetoond. Echter de frequentie van deze genomische integratie is extreem laag en vele malen lager dan de frequentie van spontane gen-inactiverende mutaties. Hierdoor is het risico op (insertionele) mutaties door integratie vele malen kleiner dan het natuurlijk risico op spontane mutaties. Nieuwe vectoren die zich richten op het verhogen van nucleaire import, integratie, (episomale) replicatie of die sequenties bezitten waarvan bekend is dat ze genomische integratie bevorderen, zouden het risico op integratie in somatische cellen kunnen verhogen. Voorts is integratie van plasmide DNA in kiembaancellen (verticale transmissie) alleen aangetoond na directe injectie in de gonaden. Met uitzondering van deze zeer uitzonderlijke toepassing is het risico op verticale transmissie verwaarloosbaar klein.
Naakt DNA kan ook in het menselijk lichaam terechtkomen door de consumptie van bijvoorbeeld met DNA gevaccineerde dieren. Het blijkt dat oraal toegediend DNA systemisch terug gevonden kan worden, maar dit resulteerde niet in de expressie van het coderende gen of tot verticale transmissie. Uit een onderzoek met stieren die een intramusculaire DNA vaccinatie hadden ondergaan bleek daarnaast dat de hoeveelheid residueel plasmide DNA in de spieren van deze dieren voor consumptie extreem klein was. Het risico van DNA transmissie door de consumptie van dieren lijkt dan ook verwaarloosbaar klein. Ook als grote hoeveelheden plasmide DNA direct aan patiënten worden toegediend is het risico minimaal. Minder dan 0.1% zal in de bloedbaan terecht komen en komt daar

niet tot expressie. Hierdoor zal het risico op genomische integratie bij orale toediening kleiner zijn dan bij een intramusculaire toediening.
De aanwezigheid van virale sequenties vergroot het risico op recombinatie met virussen aanwezig in het lichaam. Zeker indien het virus wat mogelijk gerecombineerd kan worden natuurlijk voorkomt in het individu dat het plasmide DNA product krijgt toegediend. Het milieurisico van een virale sequentie kan sterk verschillen en dient daarom per casus geëvalueerd te worden.

De term shedding wordt gebruikt voor het onbedoeld verspreiden van naakt DNA in de omgeving na toediening in de patiënt. Shedding is niet in klinische trials waargenomen en lijkt onwaarschijnlijk wanneer naakt DNA wordt geïnjecteerd in steriele lichaamsholtes of organen. Wanneer shedding toch onverhoopt plaats zou vinden, zullen de vrijgekomen hoeveelheden DNA zeer klein zijn

Bacteriën in het lichaam zouden in principe naakt DNA kunnen opnemen na (orale) toediening. Bacteriën buiten het lichaam zouden plasmide DNA kunnen opnemen na shedding of na een andere wijze van onbedoelde introductie in het milieu. Deze opname kan bevorderd worden door bacteriespecifieke opnamesequenties. Na eventuele bacteriële opname zal er echter alleen retentie van het plasmide DNA plaats vinden wanneer de aanwezigheid van het plasmide een selectief groeivoordeel voor de bacterie oplevert. Selectie kan alleen plaats vinden als het plasmide codeert voor een groeibevorderend eiwit of resistentie biedt tegen een antibioticum dat gelijktijdig aanwezig is in het lichaam of milieu. Als deze sequenties afwezig zijn zal er geen groeivoordeel voor de gastheer optreden en is het milieurisico verwaarloosbaar klein.
Nieuwe vectoren die geen sequentie voor antibioticumselectie bevatten of waarbij deze sequentie wordt verwijderd na plasmide productie bestaan en worden zelfs al klinisch toegepast. Deze ontwikkelingen zullen het veiligheidprofiel van naakt DNA nog verder verbeteren. Om de opname van plasmide DNA in menselijke cellen te verbeteren, wordt veel onderzoek verricht naar nieuwe toedieningstechnieken en naar het koppelen van DNA aan synthetische dragers. Theoretisch bestaat de mogelijkheid dat deze nieuwe technieken ook opname van plasmide DNA in bacteriën, bijvoorbeeld aanwezig op de huid bij intradermale toediening, verhogen. Dit zal echter geen verhoogd milieurisico met zich meebrengen omdat de vermeerdering en verspreiding na bacteriële opname ook dan nog steeds afhangt van het insert en resistentiegen in het plasmide.

Concluderend is het milieurisico voor veel van de gebruikte naakt DNA vectoren verwaarloosbaar klein. Er is geen grond om te veronderstellen dat nieuwe synthetische dragers en toedieningstechnieken dit risico zullen verhogen. Gebaseerd op het vorige COGEM advies (CGM/041223-02) suggeren wij om de huidge 3 veiligheidscategoriën te vervangen door 1 categorie van pDNA sequenties met een mogelijk verhoogd milieurisico (zie Tabel I). Alhoewel het absolute risico van deze sequenties waarschijnlijk erg laag is, bestaat er op dit moment geen data om dit milieurisico daadwerkelijk af te tonen. Daarom dient het milieurisico van deze catagorie vectoren per casus geëvalueerd te worden. De tweede categorie bevat alle andere vectoren waarvoor enkel een meldingsplicht noodzakelijk is.

Tabel I: naakt DNA vectoren die mogelijk een verhoogd milieurisico met zich mee brengen.

Criteria	**Mogelijk effect**
Inserts coderend voor veelgebruikte antibiotica of groeibevorderende eiwitten.	Verhoogde bacteriële retentie
Integrerende vectoren (transposons)	Verhoogde kans integratie somatische cellen
Nucleare targeting sequenties	Verhoogde kans integratie somatische cellen
(Episomale) replicatiecompetente vectoren	Verhoogde kans integratie somatische cellen
Integratie bevorderende sequenties (VDJ recombinatie signaal sequencties, mini satellieten, Alu elementen, integrases, recombinatie signalen, intact HPV E6+E7)	Verhoogde kans integratie somatische cellen
Virale sequenties (uitzondering CMV promoter en polyadenylerings signaal)	Verhoogde kans virale recombinatie en/of activatie
Directe injectie gonaden	Verhoogde kans integratie kiembaancellen

2 Summary

Naked DNA or plasmid DNA (defined in this report as pDNA administered via a non-viral method) is frequently used as vector in DNA vaccination and gene therapy applications. Plasmid is a ring of extrachromosomal DNA, which can normally be found in bacteria. By using recombinant DNA techniques a gene of interest, the so-called insert, can be introduced in the plasmid. In order to obtain expression of this insert in eukaryotic cells, plasmids contain promoter and polyadenylation sequences. In addition, an "origin of replication" and often an antibiotic resistance sequence are inserted in the plasmid backbone to induce replication in the production cell *E.coli* and to allow selection for retention. Naked DNA has a hypothetical environmental risk, which includes the risk for the creation and spreading of new genetically modified organisms (gmo's). In theory, new gmo's can be created upon the uptake of naked DNA by somatic cells, germ line cells, viruses or bacteria. The previous COGEM report from 2004 showed that the environmental risk of naked DNA is minimal. In this report COGEM advised to divide naked DNA vectors for the use in humans into three categories, based on their environmental risk. This study functioned as starting platform for the current report. In the past years, several strategies to improve the low transfection efficiency of naked DNA compared to viral vectors have been published. This research can be divided into the design of 1.) improved expression vectors, 2.) synthetic carrier systems and 3.) physical and mechanical delivery techniques.
In this report, the current environmental risk of naked DNA is reevaluated and the effect of new developments on the potential environmental risk is assessed.

Integration of naked DNA in the genome of somatic cells upon administration can occur and has been shown in experiments. Nevertheless, the frequency of integration is extremely low and far below the frequency of spontaneous gene-inactivating mutations. Therefore, the risk of insertional mutations due to integration is several folds lower than the natural risk of spontaneous mutations.
New vectors that aim to increase nuclear import, integration, (episomal) replication or that contain sequences known to increase genomic integration can increase the risk of genomic integration into somatic cells. The integration of pDNA in germ line cells (vertical transmission) has only been shown upon direct injection into the gonads. With the exception of this rare application, the risk of vertical transmission is negligible.
Naked DNA can also enter the body upon consumption of animals injected with plasmid. Orally administered plasmid can be found back systemically, but this will not result in germ line integration or expression of the insert. In addition, it has been shown that the residual levels of plasmid DNA in intramuscularly vaccinated beef bulls are extremely small.. Therefore, the risk of DNA transmission via the consumption of vaccinated animals is negligible. The direct oral administration of plasmid DNA also has a low environmental risk. Only a very small amount (<0.1%) of orally dosed plasmid will reach the circulation, without expression of the insert. Therefore the risk of genomic integration will be lower for oral administration in comparison to an intramuscular injection.

The presence of viral sequences can increase the chance of viral recombination, especially when the virus susceptible for integration can be present in the injected individual. The environmental risk of viral sequences can differ substantially and should therefore be evaluated case-by-case.

Bacteria inside the body could potentially take up naked DNA upon administration. Bacteria outside the body could potentially take up naked DNA upon shedding or via other routes of environmental

release. Shedding has not been shown in the clinic and seems unlikely when naked DNA is injected into sterile body cavities or organs.

If released into the environment, naked DNA can be taken up by microorganisms. Bacterial uptake can be increased by bacteria specific uptake sequences. Once taken up by bacteria, retention will only take place if the presence of the plasmid provides a growth advantage for the bacteria. Selection only occurs when the plasmid encodes for a growth-stimulating molecule or provides resistance against an antibiotic that is present inside the body or environment. When such sequences are absent, no growth advantage is present, resulting in a negligible environmental risk.
Novel vectors that do not contain a sequence for antibiotic selection or in which this sequence is removed upon production are present and have already been used in the clinic. These developments will increase the environmental risk profile of naked DNA. In order to increase the cellular uptake of pDNA, novel carriers systems and delivery techniques are being developed. Those novel developments could potentially increase the uptake of pDNA in bacteria, for example when bacteria are present on the skin upon intradermal administration. This will not result in an increased environmental risk since replication and spreading upon bacterial uptake depends, also in this situation, on the insert and resistance gen present in the plasmid.

The environmental risk of the majority of naked DNA vectors is negligibly low. There is no reason to assume that the described novel synthetic carriers or delivery techniques will increase this risk. Based on the previous COGEM advice (CGM/041223-02) we suggest to simplify the three-category procedure into one category of pDNA sequences that might have an increased environmental risk (see Table I). Although the absolute risk of these sequences might be very low, there is currently no data available to refute the potential risk, which thus requires a more extensive risk assessment before clinical application. The environmental risk of the category of vectors should be evaluated case-by-case, The second category contains all other vectors that only demand a duty to report.

Table 1: naked DNA vectors that might have an increased environmental risk.

Criteria	Possible effect
Inserts encoding commonly used antibiotics or growth-stimulating proteins.	Increased bacteria retention
Integrating vectors (transposons)	Increased risk integration somatic cells
Nuclear targeting sequences	Increased risk integration somatic cells
(Episomal) replication competent vectors	Increased risk integration somatic cells
Sequences increasing integration (VDJ recombination signal sequences, minisatellites, Alu elements, integrases, recombination signals, intact HPV E6+E7)	Increased risk integration somatic cells
Viral sequences (except CMV promoter and polyadenylation-signal)	Increased risk viral recombination
Direct injection gonads	Increased risk integration germ line cells

3 Introduction

"Naked" DNA, which is defined as plasmid DNA (pDNA) administered via non-viral delivery methods, is extensively used as a vector to induce transgene expression in the recipient. This provides several advantages compared to viral vectors like retro-, adeno- and adeno-associated viruses that are used for gene therapy[1]. pDNA is relative easy to manufacture, is stable during storage, can contain large inserts and is safer compared to viral vectors in terms of the risk for genomic integration and adverse reactions. The lower transfection efficiency/rate of naked DNA is considered as the most important disadvantage in comparison to viral vectors. pDNA has been tested in several (pre-)clinical applications to induce expression of antigens (DNA vaccination) or therapeutic proteins (such as Vascular Endothelium Growth Factor[2], Erythropoietin[3] or cytokines[4]). Table II provides an overview of clinically tested constructs. At this moment, no naked DNA product has been registered for use in humans. Nevertheless, four DNA products have recently been registered on the veterinary market (see Table III).

In general, naked DNA is considered a safe method of gene therapy compared to viral vectors, with low risks for both patient and environment. After administration or upon release in the environment (shedding), DNA can theoretically be taken up by somatic cells, germ line cells, viruses or bacteria, leading to the unwanted formation of genetically modified organisms (gmo's). These newly formed gmo's can spread and multiply in the environment. The environmental risk of naked DNA is defined as the chance on the formation of a new gmo's multiplied by the hazard of these newly formed organisms.

Both the EMA and the FDA provide guidelines for the use of pDNA for DNA vaccination. The Note for Guidance of the EMA (DNA vaccines non-amplifiable in eukaryotic cells for veterinary use, CVMP/IWP/07/98) discusses safety issues that should be addressed, but does not specifically mention environmental risks. In addition, the EMA provides a guideline for the risk assessment of gene therapy medicinal products (EMA/CHMP/GTWP/125491/2006). This guideline does not consider shedding by itself as an adverse effect. Only if shedding has significant consequences, for example when a plasmid provides growth advantage, it will lead to an increased risk. In addition, since the use of naked DNA can result in the formation of gmo's, Directive EC 2001/18 by the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms is applicable for the evaluation of naked DNA protocols. This directive describes that the risk for patient and environment should be accurately assessed on a case-by-case basis by a risk assessment.
The Considerations for pDNA Vaccines for Infectious Disease Indications from the FDA also evaluates safety issues including biodistribution, persistence and integration analysis. In this guideline the environmental risks of the use of naked DNA is not specifically addressed.
In the Netherlands, gene therapy protocols with naked DNA are assessed by the Ministry of Housing, Spatial Planning and the Environment, which is advised on the environmental consequences by The Netherlands Commission on Genetic Modification (COGEM).

A previous literature study by Schalk et al. [5], which was initiated by COGEM, showed that this environmental risk of naked DNA is extremely low. Based on this study, the COGEM (CGM/041223-02) advised the Ministry to classify DNA vectors in three categories based on their risk. The COGEM

suggested that low risk pDNA constructs only demand a duty to report. The previous three step procedure, advised by the COGEM is shown in Table IV.

Table IV risk groups previous COGEM advice

Group	Advice	Criteria
I	Environmental safety assessment according present procedure (besluit GGO)	• naked DNA is replication competent in eukaryotic cells; • naked DNA can become part of viruses, for example via the presence of viral packaging signals (o.a. Ψ); • naked DNA can efficiently be taken up by bacteria, for example by the presence of bacterial DNA uptake sequences (DUS), like the *Neisseria* specific DUS 5'- GCCGTCTGAA-3' and the *H. Influenza* DUS 5'-AAGTGCGGT-3'.
II	Simplified environmental safety procedure	• non-transferable plasmids with viral sequences, with the exception of CMV promoter and a polyadenylation-signal; • non-transferable plasmids containing sequences with the object to increase the recombination of naked DNA (or parts of it) with the genome or to increase the integration of naked DNA (or parts of it) into the genome. Examples are recombination signals of the Hepatitis B virus, retroviral LTR, bacteriofaag integrases, VDJ recombination signal sequences, minisatellites and Alu elements. • Non-transferable plasmid with protein-encoding sequences with transformable or oncogenic properties.
III	Duty to report, monitoring shedding	• Non-transferable plasmids based on an origin of replication (ori), present in the plamids pBR322, pUC (ColE1 ori) and p15A (pACYC-series plasmids) with a in prokaryotic active kanamycine resistance gene. • Non-transferable plasmids with an functional CMV promoter active in eukaryotic cells, a polyA signal and the eukaryotic VEGF-2 gene.

In this report, the current environmental risk of naked DNA is reevaluated and the effect of new developments on the potential environmental risk is assessed. In the last years, several strategies have been developed to increase the transfection efficiency and performance of naked DNA. In general, the following strategies can be distinguished:

- **Optimization of the expression vector**

  The first and most widely used method to improve the potency of naked DNA is the optimization of the expression cassette of the plasmid. This can augment the stability of the produced protein, or enhance the immunogenicity in case of DNA vaccination by the introduction of extra $CD4^+$ helper epitopes[6]. Additionally, optimization of promoter, open reading frame or polyadenylation signal can increase the expression properties of the DNA, which will result in higher concentration of expressed protein[7]. Besides, several other, more specific strategies such as nuclear targeting sequences, specific promoters and integrating or episomal vectors can be used.

- **Synthetic carrier systems**

  Synthetic particle carrier systems can also be used to improve the performance of naked DNA. In this approach, DNA is encapsulated into or forms a complex with a synthetic carrier (often a polymer or lipoplex), resulting in DNA-containing particles with a size ranging from 50 nm to a few micrometers. Condensation and encapsulation of DNA into micro- and nanoparticles is

extensively used for the delivery of nucleic acids for both *in vitro* as well as *in vivo* applications [8-11].

The majority of particle formulations use electrostatic interaction between anionic phosphate groups in DNA with a cationic carrier, usually composed of building blocks with positively charged nitrogen atoms. In the complex formation process, an excess of carrier is used to condense DNA into positively charged, nano-sized particles resulting in structures with a size around a few hundred nanometers. Formulation into a particle has several advantages above the usage of naked DNA. First, DNA is protected against endonuclease-mediated degradation. It is known that naked DNA is rapidly degraded upon intramuscular, intradermal or intravenous injection [12-15]. As compared to naked DNA, pDNA-containing nano- and microparticles have been shown to exhibit an increased *in vitro* nuclease resistance [16;17] and an increased half-life when injected *in vivo* [18;19]. Besides protection against nuclease-induced degradation, encapsulation into particles has the potential to increase cellular uptake of nucleic acids, which can lead to higher transfection efficiencies. It has been shown that both a small nano-scale particle size and a positive surface charge are factors that increase cellular uptake *in vitro* [20-22].

- **Novel physical and mechanical delivery techniques.**

Several physical and mechanical delivery techniques are currently used for the administration of pDNA. A potent strategy to increase the transfection of naked DNA is electroporation (EP) [23]. EP uses short electrical pulses to destabilize cell membranes. Under optimal conditions, this will lead to the formation of transient pores, which allows the entrance of macromolecules such as DNA into the cell. It is thought that electropermeabilization is followed by electrophoretic displacement of the negatively charged DNA molecule into the cytoplasm of the cell [24]. EP is an accepted technique to increase transfection efficiency *in vitro* and *in vivo* and is already used in clinic trials [25;26].

Gene gun, also referred to as biolistic particle delivery system or Particle-mediated epidermal delivery (PMED), is a commonly used technique for the delivery of naked DNA, both in skin and muscle. Gene gun is particularly used in DNA vaccine applications, both in animals[27;28] and humans[29].

Jet injectors deliver solutions of DNA into the body with a high velocity and pressure[30]. During jet injection, this pressure determines the penetration depth of the administered DNA, i.e. in skin, tumor or muscle.

Ultrasound is a relatively new strategy for the delivery of naked DNA that has been used preclinical, both *in vitro* and *in vivo*[31-33]. This technique utilizes sound waves to reversible change the permeability of cells, similar to EP, allowing pDNA to migrate into the cells.

In addition, several new dermal delivery methods, such as tattooing and microneedles are currently under development. The intradermal tattooing of naked DNA is a strategy to deliver DNA in the upper layers of the skin (epidermis) that results in robust vaccine-specific immune responses in animals[34]. This method is currently under clinical evaluation in melanoma patients[35].

Microneedles are a relatively new delivery technique for protein and DNA vaccines. By using small needles with a size between 200 and 700µm, this technique aims to deliver drugs in the epidermal layer of the skin, an important site for the administration of vaccines.

These developments might have an effect on the environmental risk of naked DNA. Therefore, this rapport will provide an evaluation of the current environmental risk of naked DNA and will estimate how new developments can possibly influence this risk.

Table II: overview of clinically used naked DNA vectors

Application	Backbone	Resistance marker	Promoter	Polyadenylation signal	Special	Insert	Administration	Ref.
DNA vaccination	pNGVL4a	kanamycin	CMV	Rabbit beta-globin	ISS in noncoding region	HPV16E7 fused with HSP	Intramuscular injection	36
	pVAX1	kanamycin	CMV	Bovine growth hormone	-	MART-1 fused with TTFC	Intradermal tattoo	35
						PSA	Intramuscular injection	37
	pcDNA3	ampicillin/ neomycine	CMV	SV40	-	DOM-PSMA	Intramuscular electroporation	38
	pNGVL3	kanamycin	CMV	Rabbit beta-globin	ISS in noncoding region	PAP	Intradermal injection	39
						MART-1 or HBsAg	Intramuscular injection	40
	pWRG	kanamycin	CMV	Bovine growth hormone	-	Gp100 or GM-CSF	Intradermal gene gun	41
	VR4951	kanamycin	CMV	Rabbit beta-globin	-	Gp100	Intradermal or intramuscular injection	42
Immuno therapy	pBR322	kanamycin/neomycine	RSV	Bovine growth hormone	Complex with DMRIE/DOPE lipids	HLA-B7 and β2-microglobuline	Intratumoral injection	43;44
	pUMVC3	kanamycin	CMV	Rabbit beta-globin	-	Interleukin 12	Intratumoral injection + electroporation	25
	pcDNA1	ampicillin/ neomycine	CMV	SV40	Complex with DC-Chol/DOPE	HLA-A2, HLA-B13 and H-2K^k	Intratumoral injection	45
Gene therapy	pUC 118	ampicillin	CMV	SV40	-	Vascular endothelial growth factor	Intravascular or intramuscular	46;47
	pVAX1	kanamycin	CMV	Bovine growth hormone	-	Hepatocyte Growth Factor	Intramuscular injection	48;49
						human smooth muscle	Corpus cavernosum injection	50

					Maxi-K channel (*hSlo*)		
pCOR	none	CMV	SV40	Only replicated in modified production cell	fibroblast growth factor type 1	Intramuscular injection	[51]

ISS = immunostimulatory sequences, HPV= human papilloma virus, HSP = heat shock protein, MART-1 = Melanoma Antigen Recognized by T cells, TTFC = Tetanus Toxin fragment C, PSA = prostate specific antigen DOM-PSMA = helper domain derived from Tetanus Toxin fused prostate specific membrane antigen, PAP = Prostatic acid phosphatise, GM-CSF = granulocyte macrophage colony-stimulating factor RSV = Rous sarcoma virus. HBsAg = hepatitis B surface antigen, SV = simian virus

Table III: overview of naked DNA vectors used in registered veterinary products

Backbone	Resistance marker	Promoter	Polyadenylation signal	Special	Insert	Administration	Ref.
APEX-IHN	kanamycin	CMV	BGA	Vaccine for salmon	Infectious haematopoietic necrosis virus glycoprotein	Intramuscular injection	[52]
LifeTide-SW5	neomycine	SPc5-12 synthetic promoter	unknown	Increase swine reproductivity	growth hormone releasing hormone	Intramuscular injection	[53]
West-Nile Innovator DNA	ampicilin	CMV	Bovine growth hormone	West Nile virus vaccines for horses	prM and E proteins of West Nile virus	Intramuscular injection	[54]
Merial DNA vaccine	kanamycin	CMV	unknown	Melanoma DNA vaccine for dogs	Tyrosinase	Intramusclar jet injection (Biojector)	[55]

4 Genomic integration in somatic cells

One of the major concerns of using naked DNA in the clinic is the potential risk of integration in the host genome of somatic cells. Genomic integration can occur during random or homologous recombination and might lead to the activation of oncogenes or the inactivation of tumor suppressor genes. Furthermore, integration into the genome of a cell can lead to an increased environmental risk. When a plasmid is integrated into the genome, the risk of transmission to another individual or to a virus present in the host cell can possibly be increased since the plasmid becomes permanently present in the host cell. Therefore, somatic integration is relevant for assessing both patient and environmental risk of naked DNA.

In risk-assessments, it is generally accepted to compare the integration rate of a pDNA with the spontaneous mutation frequency of genes. Although this mutation rate varies largely between genes, individuals and external factors (like smoking or UV exposure), an estimated value of $2x10^{-6}$ spontaneous gene-inactivating mutations per gene is most used for the comparison[56]. This number is based on a study by Cole et al., in which the mutation frequency of three genes in circulating cells was determined in several hundred volunteers[57].

Several pre-clinical studies determined the level of genomic integration upon the injection of pDNA and are discussed below:

- A study by Pal et al. determined the toxicity of intradermal and intramuscular injected pDNA in rabbits. Animals were injected with a mixture of 6 plasmids, and received maximal 4 intramuscular doses (on a monthly dose scheme) of 7.2 mg each or 4 intradermal doses of 3.6 mg each. No adverse effects were observed in the injected animals. pDNA could be detected at the injection site by PCR, 64 days upon administration. Subsequently, this study determined the amount of pDNA associated with genomic DNA. First, total DNA was extracted from the injection site and purified with three rounds of agarose gel purification to separate the high-molecular-weight fraction of genomic DNA from pDNA present in the cytosol. The presence of pDNA was then determined with quantitative PCR in the high-molecular-weight fraction. This strategy is widely used in integration determining experiments and is a surrogate marker for genomic integration, since pDNA could also be associated with genomic DNA without being integrated. In this particular study, no pDNA could be detected in the high-molecular-weight fraction [58]. The used PCR method had a sensitivity of 30 copies per 1 µg of genomic DNA. This 1 µg corresponds with approximately 150,000 genomes. Knowing that there are 2.2×10^4 genes per genome, 30 integration events per microgram of genomic DNA would be equivalent to 9×10^{-9} mutations per gene. Therefore, the worst-case mutation rate in this study is at least 200 fold lower than the spontaneous mutation rate of $2x10^{-6}$.

- Manam et al. studied the toxicity of DNA vaccines in mice (max dose 160 µg, once or twice administered) and guinea pigs (max dose 1.4 mg, one injection)[59]. This study showed low levels of pDNA associated with high-molecular-weight genomic DNA upon intramuscular injection. Around 1 copy pDNA was associated with 1 µg of genomic DNA. In case all the detected pDNA was integrated this corresponds with a maximum of 3×10^{-10} mutations per gene. The integration level was independent of adjuvant (aluminum phosphate) or administration method (jet injection or normal injection). Finally, injection of a construct encoding HPV16 E7, a protein that is known to enhance pDNA integration *in vitro*, did not result in an increased transformation in mice[59].

- A study by Martin et al. measured the genomic integration of a malaria pDNA vaccine upon a single intramuscular injection of 100 µg in mice, using PCR. This study used the same protocol for the detection of pDNA integration as the study by Pal et al. and showed that pDNA integration is at least 200 times lower than the spontaneous mutation rate of $2x10^{-6}$ per gene[60]. Older literature, that used less sensitive methodology, already confirmed that the mutation rate upon intramuscular injection of a similar amount of DNA in mice is at least 3 fold lower than the spontaneous mutation frequency[56;61].

- A study by Wang et al. determined integration upon intramuscular injection (with or without EP) of 50 µg pDNA into mice. In this study the amount of pDNA associated with high-molecular-weight genomic DNA was approximately 250 copies/µg high-molecular-weight DNA, measured 16 weeks after administration. In the worst case of complete integration, this mutation rate is still 25 times below the spontaneous mutation rate in mammalian cells. This study also showed that EP increased pDNA levels associated with genomic DNA by a factor of 15 compared to a single injection (which showed residual pDNA levels below 16 copies/ µg genomic DNA). To confirm pDNA integration (since pDNA associated with high-molecular-weight DNA is a surrogate marker), a sensitive repeat-anchored integration capture (RAIC) PCR was developed. With this assay, four independent and random integrations could be detected[62]. This was the first study that definitively demonstrated the integration of pDNA into genomic DNA following injection *in vivo*, although at levels much lower than the frequency of spontaneous gene-inactivating mutations.

- The study by Sheets et al. showed that over a course of 2 months, only a small fraction (10-20%) of animals (mice) had a detectable amount of DNA left at the intramuscular injection site (injected dose 100 µg). Integration was not observed in animals injected intramuscular by jet injection (Biojector 2000) or by needle and syringe[63]. Since the lower limit of detection was five copies of pDNA per 1 µg of high-molecular-weight mouse gDNA, the integration rate was at least 1000 fold lower than the spontaneous mutation rate in the mammalian gene.

These studies showed that genomic integration of the pDNA can be detected in pre-clinical experiments, often at levels around the lower limit of detection of the PCR methods used. It is expected that improvement of current PCR techniques will lead to the improved detection of genomic integration. Nevertheless, it should be stressed that in all studies currently published, the detected incidence of genomic integration events was far lower than the level of spontaneous mutation in mammalian genes. This was also observed upon intramuscular injection followed by EP, which normally results in the highest levels of cellular uptake and thus the highest risk of genomic integration.

The current FDA guidance for industry pDNA vaccines (version November 2007, www.fda.gov) recommends that integration studies be only warranted when pDNA persists in any tissue of any animal at levels exceeding 30,000 copies per µg of host DNA. At that level, the "worst case" integration frequency is at the same level as the spontaneous gene-inactivating mutation frequency.

Moreover, the probability that a random integration occurs at a growth-regulatory gene and thus initiates tumor growth is low. Therefore, many integration events could be innocuous. Multiplying the low levels of integration observed with the low probability of interfering with growth-regulatory genes

results in an extremely low risk of oncogenesis by random integration of naked DNA into the genome of the recipient.
The environmental risk of genomic integration is even much lower than the patient risk. Since the integration rate in somatic cells is so low, it is highly unlikely that a recombined somatic cell is shedded into the environment, taken up by another individual, followed by a new integration of the original plasmid in the recipients genome. In addition, it is probably much more difficult for a integrated plasmid to recombine with a virus than when the same plasmid is present in the cytoplasm.

4.1 Effect of novel vectors on genomic integration

Several specific sequences are associated with an increased risk of genomic integration. Examples of such sequences are VDJ recombination signal sequences (RSS), which are normally involved in the recombination of genes encoding immunoglobulins or T-cell receptors[64]. Other examples are minisatellites and Alu elements (short, tandemly repeated sequences) and viral integrases and recombination signals [65;66]. Such sequences should be used with caution.

The passage of DNA across the nuclear membrane is probably the major cellular hurdle towards gene expression, especially when cells are slow or non-dividing. Several sequences are utilized to increase nuclear import in cells. A 72-bp repeat of the Simian virus 40 (SV40), is often used to increase nuclear localization[67]. It is thought that this sequence binds to nuclear localization signals (NLS) containing transcription factors that increase nuclear import. The SV40 nuclear targeting sequence is successfully used *in vivo* to increase gene expression in muscle (20 fold)[68] and endothelial cells (10-40 fold)[69]. Several other sequences have been identified to increase nuclear uptake, such as the 10-bp nuclear factor kappa B site[70]. Furthermore, Epstein-Bar Virus (EBV) based plasmids have been associated with an increased nuclear transport[71]. Although it has never been studied, it cannot be excluded that an increased nuclear import results in a higher risk of genomic integration.

Transposons are discrete segments of DNA that can be used to integrate transgenes into the genome of host cells[72]. Naturally, these gene elements are present throughout the genome, flanked by inverted terminal repeats (ITRs). ITRs are the binding sites for transposase and allow transposition of each gene positioned between ITR sites by a ‘cut-and-paste’ mechanism. Synthetically derived transposons, like Sleeping Beauty or Piggybac can be used to insert a gene of interest, flanked by ITRs, in the genome of a target cell[73;74]. The expression of transposase is required for transposition and can be achieved by i.) inserting a transposase gene in the same vector, ii.) expressing it in a separate plasmid, iii.) using mRNA encoding transposase or iv.) using recombinant transposase. The major advantage of transposon-based vectors is their ability to generate long-term expression without using a viral vector. Sleeping Beauty-based systems are successfully used to transduce peripheral human lymphocytes with T cell receptor genes[75]. Since these constructs are aiming to generate stable integration in the genome of the target cell, it is logical to be extremely careful with injecting such constructs directly into patients, since random integration cannot be excluded[72].

Another approach to increase pDNA retention is the use of episomal vectors that replicate extrachromosomally upon cell division. Episomal plasmids are typically based on sequences from DNA viruses, such as BK virus, bovine papilloma virus 1 and Epstein-Barr virus (EBV). An advantage of these systems is that replication leads to accumulation of the pDNA in the nucleus. This increased number of pDNA copies leads to increased levels of target gene mRNA and protein expression[76]. Since integration in the host genome can occur[77] and is increased due to the intracellular

replication, we consider all replicating vectors to be associated with an increased risk of genomic integration.

In addition, it is known that constructs encoding human papilloma virus (HPV) type E6 and E7 proteins can increase pDNA integration *in vitro*. Although, the integrating effect of E7 protein was not observed upon intramuscular injection of an E7 construct in mice[59], intact E6 and E7 protein should be used with caution. The potential transforming effect of E6 and E7 can be easily circumvented without inhibiting the immunogenicity (which is wanted for vaccine purposes) by using a construct encoding the shuffled variant of the protein[78].

4.2 Effect of delivery technique on genomic integration

Novel delivery techniques often aim to improve the transfection efficiency of naked DNA. However a higher intracellular pDNA accumulation can also increase the probability of genomic integration.
In skeletal muscle, EP increases transgene expression of a single injection a 100- to 10.000-fold [79]. As a result, intramuscular injection followed by EP is currently the technique that results in the highest and longest levels of gene expression compared with other delivery techniques or synthetic carriers (which increase expression upon intramuscular injection by maximal 10-fold [80;81]) and thus possesses the highest risk of genomic integration.
The discussed study by Wang et al. showed that EP upon intramuscular DNA injection in mice is associated with an increased cellular uptake and an increased level of pDNA associated with high-molecular-weight genomic DNA[62]. In this study, EP increased the levels of pDNA associated with genomic DNA 15-fold compared with a single injection. Nevertheless, the worst-case integration frequency was still 25 times lower in this situation, than the spontaneous gene-inactivating mutation rate. pDNA persistence was not increased in murine skin, upon intradermal injection combined by EP compared with injection without EP. Nevertheless, this publication only studied the general persistence and not pDNA associated with high-molecular-weight DNA[82].
A recent study assessed pDNA persistence and genomic integration upon intramuscular pDNA injections in macaques, with and without EP. It was shown that EP was associated with a decreased level of pDNA persistence at the intramuscular administration site, with no increase in pDNA associated with high-molecular-weight DNA (measured 70 days after injection) [83]. For all injections, with or without EP, the level of pDNA associated with genomic DNA was between 50 and 100 copies/µg genomic DNA, which is at least 70-fold lower than the spontaneous mutation rate. This observation suggests that an increased transgene expression due to EP does not necessarily have to result in a higher risk of genomic integration.
It is currently unclear why increased levels of pDNA associated with genomic DNA are observed in mice and not in macaques. A difference in clearance of the transfected myocytes could be a possible explanation. More importantly, these studies demonstrated that for the delivery strategy with the highest levels of transgene expression (intramuscular injection with EP), the integration rate is still far below the spontaneous mutation frequency.

4.3 Effect of synthetic carriers on genomic integration

Since synthetic carriers increase cellular uptake of naked DNA and might therefore increase the level of genomic integration at the injection site. The integration frequency of synthetic carriers has been described in two publications of Vilalta et al. The first publication described the persistence and integration of pDNA formulated with poloxamer (a synthetic polymer) upon intramuscular injection in mice and rabbits[84]. The intramuscular persistence of pDNA (80 µg) formulated with poloxamer was 8-

fold higher when compared to naked DNA, measured 60 days post-injection in mice. To assess whether this increased tissue persistence resulted in increased levels of genomic integration, residual pDNA associated with the high-molecular-weight fraction of DNA extracted from the injection site, was measured with Q-PCR in rabbits (dose of 5 mg pDNA). No residual pDNA could be measured at the injection site in animals that were administered with poloxamer/DNA complexes. The lower limit of detection of the used PCR method was 20 copies of plasmid/µg of genomic DNA.

Similar results were found in a second publication by the same group in which persistence and genomic integration of pDNA formulated into DMRIE:DOPE liposomes was studied upon intramuscular injection in rabbit (dose of 1 mg pDNA)[85]. The DNA clearance of liposome-formulated pDNA from the injection site was similar as for poloxamer-formulated pDNA. The levels of genomic integration were measured 64 days post-injection with the same procedure and Q-PCR and were below the lower limit of detection.

These studies showed that the worst-case integration frequencies of liposome- and poloxamer-formulated pDNA are at least 300-fold below the spontaneous gene-inactivating mutation frequency. Since the levels of integration were below the lower limit of detection, it cannot be excluded that synthetic DNA carriers increase the risk of genomic integration. However, if this occurs, the integration frequency is still far below the spontaneous gene-inactivating mutation frequency, resulting in a low risk of genomic integration for these systems.

5 Genomic integration in germ line cells (vertical transmission)

Genomic integration into germ line cells can potentially lead to vertical transmission of pDNA to offspring.
Naked DNA can be detected in gonads, shortly upon intramuscular injection in mice. Nevertheless, these DNA levels drop rapidly below detection limits. Furthermore, in both testis and ovaries, detected pDNA was present extrachromasomally, as confirmed by agarose gel separation[59]. A study in swines also showed detectable levels of pDNA in ovaries upon intramuscular injection, which were below the limit of detection after 1 week[86]. Another study showed that no pDNA could be detected by PCR in spleen or liver tissue harvested from F1 offspring of vaccinated mice, showing that the injected pDNA was not integrated in the germ line[87].
An intracavernous injection of pDNA does not lead to detectable levels of pDNA in semen of patients[88]. Nevertheless, a study in mice showed that direct injection of a pDNA encoding EGFP-gag into mouse ovaries lead to successful integration into the genome of the oocytes, and retained its function in replication, transcription and translation following at least one mitotic division in embryos[89]. This observation stresses that vertical transmission can be a risk when pDNA products are directly injected into the gonads. Therefore, the risk of vertical transmission should be carefully evaluated when pDNA is directly administered into the reproductive tract. The patient reproductive potential could then be a determining factor in evaluating the potential risk of vertical transmission.

Vertical transmission can be increased when using novel vectors that aim to improve nuclear localization, integration, replication or are associated with a higher risk of integration. Nevertheless, this increased risk is only present if the construct is directly injected into the gonads, which is the only route of administration for naked DNA that can lead to genomic integration of germ line cells.

A relevant study by Nabel et al. studied the vertical transmission of pDNA/DC-cholesterol complexes (1 µg pDNA) upon i.v. injection or arterial infusion in mice, rabbits and pigs. No pDNA could be detected with PCR in the testis or ovaries from any of the treated animals, 21 days after injection[90]. The sensitivity of the used PCR assay was estimated to be 1 copy per 10^3 to 10^5 genomes. Therefore, the risk of vertical transmission of a systemically administered non-viral carrier systems can be considered low, similar to naked DNA. The only exception is a direct injection of synthetic carriers into the gonads, which might lead to an increased integration into germ line cells[89].

Vertical transmission can be increased upon direct administration of naked DNA into the gonads by new delivery techniques. EP has already been used to increase naked DNA uptake in the testis of mice and hamsters [91;92]. Using this strategy, expression of the transgene could be detected in sperm for as long as 60 days following gene transfer[92], suggesting integration in the genome of germ line cells.

5.1 Plasmid uptake via the oral route

The consumption of DNA vaccinated animals or the oral intake of pDNA can be another source of plasmid that can be integrated into germ line cells or somatic cells. Although the majority of digested pDNA will be degraded in the stomach, naked DNA fragments can be tracked in several organs (leukocytes, spleen and liver) upon oral administration, probably transported through the intestinal wall[93]. Only a very small fraction of the administered pDNA dose (≤0.1%) can be detected in the

blood of the recipient between 2 and 8 hrs upon administration. The researchers suggest that this small fraction of DNA is protected from degradation via the formation of DNA/protein complexes. DNA fragments between 194 and 976 bp in length were identified by PCR in the blood of DNA-fed animals.

Although the presence of DNA can be measured in the circulation upon oral intake, it was also shown that the oral intake of GFP DNA in mice (50µg for 21 days) does not lead to detectable expression of GFP in blood liver, spleen or intestinal wall in the recipient (measured by RT-PCR)[94].

Orally administered DNA can also be found back in offspring of pregnant mice, by passage over the placenta [95]. The maximal fragment length in fetal DNA, as determined by PCR, was about 830 bp. In none of the studied animals all cells of the fetus were transgenic for the foreign DNA. This distribution pattern argues for a transplacental pathway rather than for germ line transmission. These results suggest that the administration of pDNA to pregnant women should be done with high caution.

In a follow-up study of the same group, mice were continuously fed with 50µg of GFP DNA daily for eight generations. In each of the generations, the presence of GFP fragments was analyzed by PCR in DNA extracts from the tail, liver and spleen. In none of the examined animals (12 per generation), GFP encoding DNA fragments could be detected in the tissue[94]. This suggests that there is no support that orally administered DNA can be transferred to second generations via germ line integration. Apparently, the germ line is protected from foreign DNA, which is constantly entering our circulation upon oral intake of food.

The intake of plasmids via the consumption of meat from cattle, treated with a DNA product, could be another source of DNA transfer. The retention of pDNA has been studied in DNA vaccinated beef bulls (500 µg per dose). Quantitative PCR analysis on day 242-292 after the last intramuscular administration showed that residual pDNA could only be found back at the injection site. The highest pDNA level was detected in the group administered with pDNA/liposome complexes. Lower residual DNA levels were found upon the administration of naked DNA by intramuscular injection or electroporation. No pDNA was detected in biopsy from distant muscles and draining lymph nodes in all groups[96]. The maximum amount of 290 copies pDNA / 500 ng genomic DNA, found per PCR reaction roughly represent an almost undetectable intake of pDNA (400 fg/g muscle tissue) for consumers. This is approximately 2×10^9 fold lower than the amount of genomic DNA present in meat. Residual pDNA in native state will hardly be found at a measurable level following further meat processing.

Although pDNA can reach the circulation upon oral intake, only very low amounts of the administered dose will reach the circulation. No germ line transmission has been observed upon the oral intake of pDNA. In addition, the oral intake of pDNA does not lead to expression of the encoded gene in the recipient. As a result, the risk of genomic integration in somatic cells is most probably lower upon the oral administration of pDNA when compared to an intramuscular injection, which normally leads to the highest expression levels and genomic integration rates.

6 Recombination with viruses

Upon administration, pDNA can theoretically be recombined with viruses inside the individual leading to the formation of new infectious viruses. This risk is increased when viral sequences are present in the plasmid. The most frequently present viral sequence in plasmids is the CMV promoter (see Table II). Although CMV is abundantly present in the population (in 2005, 41% of pregnant women in the Netherlands tested CMV positive in the Netherlands [97]), viral recombination events have never been reported for CMV promoter containing plasmid.

Recombinant viruses can be generated *in vitro,* by the co-transfection of a transfer plasmid together with viral DNA into a host cell line[98], which will lead to the integration of the plasmid into the viral DNA. In a similar strategy, a transfer plasmid is transfected into virally infected host cells to induce integration of the plasmid into the virus[99]. Retroviral plasmid vectors, such as pMX, are providing the viral packaging signal, transcription and processing elements for the production of retroviruses upon transfection into a packaging cell line. All such plasmids, which are normally used for the *in vitro* generation of new recombinant viruses might have an increased environmental risk, since viral recombination is theoretically also possible upon *in vivo* administration.

The previous COGEM report (CGM/041223-02) positioned sequences that improve viral insertion, with long terminal repeat (LTR) and Ψ packing signals as examples, into the highest risk group (see Table IV). In addition, all other viral sequences are positioned in the second risk group, except for the CMV promoter and polyadenylation-signal, which are both extensively used in clinical trials and considered safe.

When evaluating the environmental risk of viral sequences in plasmids, it is important to take the natural incidence of possible target viruses in the administrated species into consideration. The possible occurrence of the virus susceptible for recombination inside transfected cells will be a major factor determining the true risk of viral recombination. New developments in the field that lead to an increased cellular uptake of plasmid only lead to an increased risk of viral recombination when viral sequences are present in the plasmid. When viral sequences are present in the plasmid, the actual risk is dependent on the virus type, the encoding sequence and the possibility of survival advantage. The environmental risk of viral sequences should therefore be evaluated on a case-by-case basis.

7 Environmental release of DNA

pDNA can potentially be spread into the environment via body fluids upon injection into the body. This process is referred to as shedding. Shedding forms a potential risk, since the released pDNA can be taken up by bacteria in the environment. This uptake can lead to the formation of new genetically modified organism possibly followed by an uncontrolled spread of the plasmid.
Two studies analyzed environmental shedding of pDNA upon injection into patients. The study by Comerota et al. did not detect pDNA in urine upon intramuscular injection of patients with a construct encoding fibroblast growth factor type I[100]. In this study, single doses between 500 and 4000 µg were tested and the presence of pDNA in urine was determined via PCR. A study by Melman et al. showed that injection of naked DNA into the corpus cavernosum in the penis, did not result in detectable levels of pDNA by PCR in the semen of the patients[88]. The lower limit of quantification of the used PCR was 1 copy per µg of total DNA extracted from the semen.

Although not widely studied, the shedding of pDNA into the environment can be considered as a negligible risk, especially when pDNA is administered into a sterile body cavity.
It is known that pDNA can remain at the injection site for several months upon intramuscular injection[58;101]. However, when released into the circulation, the half-life of pDNA is extremely short. Agarose gel electrophoresis showed that the supercoiled form degrades with a half-life of 1.2 minutes in rats. The open circular and linear form had a half-life in blood of 21 and 11 minutes respectively [14].
A recent study by Gravier et al. was the first publication in which the biodistribution of pDNA was studied in larger animals (swines) upon intramuscular injection[86]. In the injected muscle, pDNA remained detectable for at least 4 weeks (using qPCR analysis, with a lower limit of detection between 34 and 91 copies per µg of extracted DNA). In addition, pDNA was also detected in liver, spleen, kidney, lung, remote muscle, lymph nodes and ovaries. The persistence in these other organs was shorter than at the injection site (for the ovaries, no persistence for longer than 1 week could be detected). Less than 10% of the injected dose reached the circulation and was rapidly degraded with a half-life of minutes.

A study by Zhang et al. studied the biodistribution of radioactively labelled pDNA upon intramuscular injection in mice. A large part of intramuscular injected pDNA (around 50%) was excreted via urine. Nevertheless, this study only measured radioactivity and not intact plasmid[102]. The cut-off size for glomerular filtration is approximately 60kD. As a result, only small fragments of DNA, smaller than 100bp, can potentially leak into the environment via urine. This length is smaller than the average size of a gene. Therefore, it is theoretically impossible that intact and bioactive circulating plasmids can leak into the environment via urine when glomerular filtration is intact. This hypothesis was confirmed by Comerata et al. who did not observe pDNA in urine of patients injected with plasmid[51]. It is unlikely that altering the nucleotide sequence will influence shedding since the physical chemical properties of the DNA molecule are not changed.

When DNA is administered on or into the skin, the potential risk of shedding may be increased (for example during DNA tattooing or microneedle application).
Intradermally administered pDNA stays detectable by PCR for more than 2 months[103]. Nevertheless, it is known that mouse skin contains endonucleases that can degrade 99% of intradermally injected DNA within 90 min, similar as for intramuscular injections[12]. In addition, human skin also showed high

DNAse activity, especially at the basal layer and the upper layers of the epidermis[104]. Therefore the risk of shedding might be lower for the intradermal route of administration than expected.

Once released into the environment, the half-life of the DNA will be an important factor determining the environmental risk. This half-life strongly depends on the location. In aqueous environments (waste water, fresh water, salt water), the half-life of DNA varies between a few minutes to several hours. In a terrestrial environment (sediments, soil), DNA can be adsorbed on soil, which will dramatically increase the half-life of DNA. Transformation-active DNA can persist in soil for weeks or months, since adsorption on soil protects DNA against nuclease-mediated degradation[105]. The long half-life on soil can lead to a deposit of DNA in the environment. This is certainly unwanted since such a deposit can increase the possibility of bacterial uptake. Nevertheless, this potential increase is not relevant when naked DNA is administered in a clinical setting.

The effect of a non-viral carrier on the risk of shedding has not yet been described in the literature. It is known that formulation into a carrier system can change the pharmacokinetics of injected pDNA upon *in vivo* injection[15;84]. In addition, the hydrodynamic radius of a DNA nanoparticle is smaller than the hydrodynamic radius of naked pDNA, which might lead to an increased risk of environmental release upon intravenous injection via urine. Nevertheless, the average size of an IgG antibody, which cannot pass the glomerulus, is 9 nm[106]. Since the smallest DNA containing nanoparticles have a size of at least 30 nm, which is too large for glomerular passage, it is not likely that non-viral carrier mediated gene therapy lead to more shedding via urine than naked pDNA when renal function is intact.

Physical and mechanical delivery techniques aim to improve uptake into target cells. When DNA is delivered into sterile tissue by these novel strategies, the risk of shedding is probably not increased in comparison to a classic injection, since the DNA is delivered into a depot inside the body. Instead, the risk of shedding might even decrease, since higher cellular uptake levels will result in lower amounts of pDNA available for environmental release.

In conclusion, shedding of pDNA is low when administered inside the body. Novel dermal delivery techniques that apply pDNA onto the skin can increase the shedding rate.

8 Bacterial uptake and persistence (horizontal transmission)

Once shed into the environment, secreted pDNA can potentially be taken up by bacteria (horizontal transmission). Theoretically, this process can lead to the formation of genetically modified bacteria and uncontrolled spread of the pDNA into the environment. But before a genetically modified bacterium is established, two major hurdles have to be taken, first the pDNA has to be taken up by the bacterium. Secondly, the presence of the pDNA should confer a survival advantage to the bacterium in order to confer retention.

In general, the uptake of naked pDNA by bacteria is low. Since the cell wall of bacteria is negatively charged, diffusion of negatively charged pDNA, with a molecular weight over 2000kD, into the cell is nearly impossible. Under laboratory conditions, naked DNA is only taken up by bacteria in the presence of $CaCl_2$[107], chelators, enzymes[105] or when mediated via electroporation [108] or PMED[105]. Nevertheless, a limited number of naturally transformable bacteria such as *Haemophilus influenzae*, *Neisseria meningitides* or *Streptococcus pneumoniae* are known[109;110]. It is thought that this natural transformation plays an important role in the genetic adaptation to the environment. It cannot be excluded that these microorganisms can take up shed pDNA as well. pDNA should preferable not contain known bacterial 'DNA uptake sequences' (DUS) that are able to preferentially transform bacteria like *Neisseria* and *Pasteurellaceae* [111].

Upon transformation, survival advantage for the bacterium is essential to maintain pDNA retention in the bacterium. Survival advantage might for example occur when the pDNA is encoding a metabolic gene or antibiotic resistance gene. Antibiotic resistance sequences are widely used in plasmids to provide selection pressure during pDNA production. Only when such selection antibiotics are present in the environment, the presence of pDNA in the bacterium will provide a survival advantage and thus facilitate pDNA retention. Kanamicin resistance genes are most extensively used in clinical grade constructs. The main rationale for this choice is the lack of hyper-sensitivity for this antibiotic in patients. Furthermore this antibiotic is sporadically used in the clinic and in veterinary medicine[112], reducing the risk for environmental selection. Nevertheless, the most commonly used kanamycin resistance genes npt-II and npt-Ia confer also resistance against neomycine[113]. Although neomycine is not widely used in clinical practice, it has a clinical value in dermal applications and is used for the treatment of hepatic encephalopathy[112]. Although pDNA with ampicilin resistance genes have been used in the clinic without any reported environmental hazards (see Table II), we advice to avoid this resistance gene in future pDNA designs. Ampicilin resistance sequences encoded for the enzyme β-lactamase, which provides resistance against all antibiotics with a β-lactam ring structure. Since this is large group of antibiotics (including amoxicillin, flucloxacillin and penicillin) which is widely used in clinical and veterinary practice, the theoretical risk of bacterial persistence of plasmids carrying ampicillin resistance is theoretically higher than the risk of plasmids carrying a kanamycin/neomycine resistance gene.

A study performed in the late 70's demonstrated the low environmental risk of pDNA handling in the laboratory. Faeces of laboratory workers who handled *E.coli* K-12 resistance to the antibioticum nalidixic acid and plasmids with multiple drug resistance markers were monitored for over 2 years. During those 2 years, 229 faecal samples and 2482 *E.coli* strains have been examined. In none of the tested samples, nalidixic acid resistance *E.coli* K-12 could be detected. During those 2 years, 26

samples of *E.coli* showed a resistance pattern that was similar to the plasmids used in experiments. Nevertheless, digestion of extracted plasmids from the bacteria with restriction endonucleases showed a different electrophoretic pattern than the plasmids used in experiments, indicating that none of the plasmids handled in the laboratory was established/maintained in the faecal flora. This study showed that the practical risk of transmission during laboratory handling is absent[114].

pDNA can also be transmitted to microorganism inside the administered individual. The gastro-intestinal (GI)-tract is the most probable location for this potential recombination since this is the only location of the body with a high density of microorganisms. Nevertheless, it is highly unlikely that DNA with its huge size and hydrophilic nature, administered into sterile body cavities or skin, will reach the GI-tract of the recipient. When pDNA can reach the GI-tract, for example by oral administration, it is not likely that plasmid-containing bacteria retain the pDNA inside the body in the absence of selection pressure, similar to the situation outside the body. A relevant study by Dalboge et al. showed that *E. coli*, containing recombinant plasmid, was only detectable in faeces of rats, one or two days after oral administration of 10^{11} bacteria[107]. When animals were continuously treated with the selection antibiotic ampicillin (for 6 days), a relatively high number of recombinants was maintained during that period. Nevertheless a significant decrease in number of recombinants was seen in these animals as well. A few days after discontinuation of ampicillin treatment no recombinants could be detected in the faeces any more. In addition, Inoue et al studied the persistence of *E. coli* carrying a pDNA with a resistance gene against aminobenzylpenicillin and kanamycin upon inoculation in the bladder of rats. When plasmid-carrying *E.coli* was incubated alone, plasmid-carrying *E.coli* was spontaneously replaced with plasmid-free *E. coli*. This study calculated that 1/100 to 1/1000 of plasmid-carrying *E.coli* loses pDNA per generation. In addition, the inoculation of plasmid-carrying *E.coli* together with plasmid-free *E.coli* resulted in an accelerated replacement of plasmid-carrying *E.coli* with plasmid-free *E.coli*. This demonstrates that the presence of a pDNA increases the generation time of *E.coli in vivo*, which will lead to an overgrowth of plasmid-carrying bacteria with plasmid-free bacteria[115].

8.1 Novel vectors lacking antibiotic resistance genes

Various selection systems are under development to eliminate the need for antibiotic selection genes, reducing the risk of horizontal transmission. The first possibility is to make use of an auxotropic production cell, in which an essential protein is excluded from the genome. By presenting this essential gene in the pDNA backbone, the pDNA provides selection and is maintained in the modified bacterial strain. Several examples of such systems are known:
The pCOR vector only replicates in a specifically engineered, arginine-deficient *E.coli.* When this deficiency is corrected by the tRNA sup Phe from the plasmid, the host cell can survive leading to pDNA replication[116]. The pCOR vector has already been used in the clinic (see Table II).
A second example is the deletion of the *glyA* gene in *E.coli*, which encodes for serine hydroxymethyl transferase, essential in glycine biosynthesis. The pDNA pQEαβrham contains the *glyA* homologous gene, allowing transformed cells to grow on minimal media without glycine supplementation or antibiotic[117]. Recently, an *E.coli* strain carrying a defect in NAD *de novo* biosynthesis was constructed by knocking out the chromosomal quinolinic acid phosphoribosyltransferase (*QAPRTase*) gene. By constructing a pDNA with the *QAPRTase* gene, expression of this gene was rescued, allowing growth of the production cell[118].

Another approach is repressor titration of engineered host cells. In this approach, an essential gene is repressed in engineered host cells. By introducing an operator (for example *lac*) in a plasmid, the essential gene is derepressed and expressed, allowing cell growth. Example of genes that are used for repressor titration are dapD, which is derepressed by the pORT pDNA which contains the *lac* operator[119], or an antibiotic resistance gene, which can be titrated by the pGM509 plasmid[120].

RNA based selection systems use endogenous RNAI/RNAII antisense regulators. In these systems, a growth-inhibiting gene is introduced in the host cell that contains an RNAII sequence in the untranslated region of the mRNA. The introduction of a pDNA encoding RNAI results in RNAI:RNAII binding, suppressing the translation of the chromosomal gene by antisense regulation[113]. Recently a vector expressing a 150 bp RNA-OUT antisense was constructed which represses the expression of a chromosomally integrated counterselectable marker (sacB). This systems allows pDNA selection on sucrose[121].

Another approach to remove antibiotic selection genes is the minicircle technique. In this strategy, the sequences of the pDNA essential for bacterial replication (as the origin of replication and antibiotic selection) are enzymatically removed upon production. The product of this enzymatic process is a smaller pDNA only encoding the essential sequences for eukaryotic expression, thereby reducing the environmental risk[122].

All the discussed approaches reduce the risk of replication in common, non-engineered, bacteria since no selection is present in these cells. These novel vectors are not only safer; they are generally smaller than a pDNA containing an antibiotic resistance marker, increasing their biologic activity.

8.2 Effect of novel developments on risk of horizontal transmission

It is known that inverted terminal repeats (ITRs), the sites that allow integration of transposon, are also present on the genome of *E.coli* and thereby increase the risk of horizontal transmission for this vector system[123]. The successful integration of a transposon requires the presence of the enzyme transposase. It is possible to insert a transposase encoding region in the same vector as the transposon element (*cis*-vector). However this approach might increase the risk of horizontal transmission, since the uptake of 1 pDNA copy can be sufficient to transform a bacterium. By using an exogenous transposase source like mRNA or purified protein, or by using a second vector which encodes the transposase (*trans*-vector), the risk of horizontal transmission for these vectors can be decreased[72]. Horizontal transmission is not increased when using vectors that aim to improve nuclear localization since bacteria do not contain a nucleus.

The positive charge of most non-viral carrier systems might facilitate an increased horizontal transmission into bacteria, since all bacteria have a negatively charged cell wall. Nevertheless, when the encapsulated pDNA provides no selective benefit to the bacteria, this potential increase in horizontal transmission will not lead to an increased accumulation of pDNA into the environment[107]. A pilot experiment described in appendix I demonstrated that the environmental risk of synthetic carriers is probably low.

In addition, since microorganisms are abundantly present on skin, dermal electroporation can facilitate an increased uptake of DNA. Electroporation can be used to transform bacteria[124], in which the efficiency of transformation is highly dependent on the field strength and pulse duration. Nevertheless,

as discussed before, in the absence of selection pressure, this pDNA will not be retained in the transformed microorganism. In addition, most clinical protocols will have a disinfection step inserted which will reduce the chance of bacteria transformation. A pilot experiment described in appendix II showed no increase in transformed bacteria upon dermal DNA tattooing in patients.

9 Conclusion

Naked DNA is widely used in gene therapy. In the past years, many researchers have tried to increase the performance of naked DNA by improving vector sequences, by formulation with a non-viral carrier or by using improved physical and mechanical delivery techniques.

The administration of naked DNA includes several environmental risks (the formation of gmo's) that should be carefully evaluated before these products can be applied to a large patient group. Based on a previous literature survey by Schalk et al. in 2004[5], the COGEM advised the Ministry of Housing, Spatial Planning and the Environment (CGM/041223-02) to use a three category procedure to estimate the risk of pDNA used for DNA vaccination. Group I includes constructs with the highest risk that should be evaluated by the Ministry via the standard procedure. Group II includes constructs with a lower risk that should be evaluated via a shorter procedure. Group III includes constructs at the lowest risk that only demand a duty to report (see Table IV for an overview). This COGEM advice served as a starting point for this study.

Based on current literature the environmental risk of the vast majority of naked DNA vectors can be considered very low and not higher than that of the approved conventional live and inactivated vaccines. Integration of plasmid DNA sequences in the genome of somatic cells has been observed, but even in the case of administration via the delivery technique that results in the highest expression levels (intramuscular injection with EP), the extend of integration is still far below the spontaneous gene-inactivating mutation rate of a somatic cell. With the exception of certain vectors or sequences that can increase integration in the genome of somatic cells, the novel developments discussed here to improve gene expression or immunogenicity have not shown any increase in the environmental risk of the use of naked DNA.

Apart from the direct injection into gonads, germ line transmission has never been observed upon other routes of administration. Adsorption and systematic distribution of pDNA upon oral administration has been observed. Nevertheless, it should be stressed that much larger quantities of viral and bacterial DNA are constantly entering our circulation via food intake with no reported environmental hazard. In addition, there is no evidence that orally administered pDNA is vertically transmitted via germ line cells or leads to the expression of the insert in body cells.

Viral sequences should be avoided since viruses susceptible for recombination may be present in the recipient, and this might lead to the formation of new viruses. The use of a CMV promoter and/or a polyadenylation-signal are considered safe based on the wide experience with these sequences in clinical trials.

Upon possible uptake by bacteria, retention of pDNA will only occur when the insert provides a growth advantage. Therefore, inserts that provide such a survival advantage (like metabolic genes and resistance genes against widely used antibiotics) should preferably be avoided.
Antibiotic resistance genes are widely used in the production of plasmids. For this process, antibiotics that are not widely used for the treatment of patients or animals have been carefully selected. In addition, the development to design plasmids in which the antibiotic resistance genes are excluded is encouraged, since this will even further lower the remote probability of horizontal transmission.

There is currently no literature available that describes the effect of synthetic vectors and novel delivery techniques on horizontal transmission. In order to fill the current gap in the literature, two pilot experiments have been performed to study the bacterial transmission of synthetic carriers and dermal DNA tattooing (see Appendices I and II). Although the results of these preliminary experiments should be interpreted with caution, they suggest that synthetic carriers shed into the environment, or the application of naked DNA via dermal tattooing do not result in an increased pDNA uptake and retention in bacteria. This is supportive of the fact that it is unlikely that these novel developments increase the environmental risk of naked DNA.

In conclusion, for the vast majority of vectors currently used, the environmental risk is negligible. Especially when realizing that we are exposed to large amounts of DNA from bacterial and viral origin in our environment and during our daily food intake. At this point it is highly questionable whether an objective measurement of such low risks will ever be possible in future research. Based on the current literature and experience with the use of naked DNA, we consider a further simplification of the current environmental regulations for the majority of naked DNA vectors justified. This is supported by the notion that thousands of researchers over the world have handled large amounts of pDNA in the laboratory over decades with no apparent special precautions and no reported toxicity or environmental hazards.

10 Advice

Based on the previous COGEM advice (CGM/041223-02) we suggest to simplify the three-category procedure into one category of pDNA sequences that might have an increased environmental risk (see Table V for a detailed overview). Although the absolute risk of these sequences might be very low, there is currently no data available to refute the potential risk, which thus requires a more extensive risk assessment before clinical application. The second category contains all other vectors that only demand a duty to report. In clinical practice, the large majority of vectors used in trials (see Table II) will fit in this last category.

Table V: naked DNA vectors associated with increased environmental risk

Criteria	Rationale
Inserts encoding commonly used antibiotics or growth-stimulating proteins.	Can possibly increased bacteria retention, since insert provide survival advantage upon bacterial uptake
Integrating vectors (transposons)	These vectors aim to integrated into the genome of the host cell, increased risk genomic integration
Nuclear targeting sequences	Increased risk integration somatic cells since nuclear accumulation is increased
(Episomal) replication competent vectors	Increased risk integration somatic cells since vector are replication competent in host cell
Sequences increasing integration (VDJ recombination signal sequences, minisatellites, Alu elements, integrases, recombination signals, intact HPV E6+E7)	These sequences are associated with an increased risk on integration in somatic cells
Viral sequences (except CMV promoter and polyadenylation-signal)	Increased risk viral recombination upon transfection of host cell
Direct injection gonads	Only delivery route which result in vertical transmission into germ line cells

References

[1] Hendrie PC, Russell DW. Gene targeting with viral vectors. Mol Ther 2005 Jul;12(1):9-17.

[2] Kusumanto YH, Hospers GA, Mulder NH, Tio RA. Therapeutic angiogenesis with vascular endothelial growth factor in peripheral and coronary artery disease: a review. Int J Cardiovasc Intervent 2003;5(1):27-34.

[3] Svensson EC, Black HB, Dugger DL, et al. Long-term erythropoietin expression in rodents and non-human primates following intramuscular injection of a replication-defective adenoviral vector. Hum Gene Ther 1997 Oct 10;8(15):1797-806.

[4] Galanis E, Hersh EM, Stopeck AT, et al. Immunotherapy of advanced malignancy by direct gene transfer of an interleukin-2 DNA/DMRIE/DOPE lipid complex: phase I/II experience. J Clin Oncol 1999 Oct;17(10):3313-23.

[5] Schalk JA, Mooi FR, Berbers GA, van Aerts LA, Ovelgonne H, Kimman TG. Preclinical and clinical safety studies on DNA vaccines. Hum Vaccin 2006 Mar;2(2):45-53.

[6] Wolkers MC, Toebes M, Okabe M, Haanen JB, Schumacher TN. Optimizing the efficacy of epitope-directed DNA vaccination. J Immunol 2002 May 15;168(10):4998-5004.

[7] Xu ZL, Mizuguchi H, Ishii-Watabe A, Uchida E, Mayumi T, Hayakawa T. Optimization of transcriptional regulatory elements for constructing plasmid vectors. Gene 2001 Jul 11;272(1-2):149-56.

[8] De Smedt SC, Demeester J, Hennink WE. Cationic polymer based gene delivery systems. Pharm Res 2000 Feb;17(2):113-26.

[9] El Aneed A. An overview of current delivery systems in cancer gene therapy. J Control Release 2004 Jan 8;94(1):1-14.

[10] Jilek S, Merkle HP, Walter E. DNA-loaded biodegradable microparticles as vaccine delivery systems and their interaction with dendritic cells. Adv Drug Deliv Rev 2005 Jan 10;57(3):377-90.

[11] Lungwitz U, Breunig M, Blunk T, Gopferich A. Polyethylenimine-based non-viral gene delivery systems. Eur J Pharm Biopharm 2005 Jul;60(2):247-66.

[12] Barry ME, Pinto-Gonzalez D, Orson FM, McKenzie GJ, Petry GR, Barry MA. Role of endogenous endonucleases and tissue site in transfection and CpG-mediated immune activation after naked DNA injection. Hum Gene Ther 1999 Oct 10;10(15):2461-80.

[13] Bureau MF, Naimi S, Torero IR, et al. Intramuscular plasmid DNA electrotransfer: biodistribution and degradation. Biochim Biophys Acta 2004 Jan 20;1676(2):138-48.

[14] Houk BE, Hochhaus G, Hughes JA. Kinetic modeling of plasmid DNA degradation in rat plasma. AAPS PharmSci 1999;1(3):E9.

[15] Houk BE, Martin R, Hochhaus G, Hughes JA. Pharmacokinetics of plasmid DNA in the rat. Pharm Res 2001 Jan;18(1):67-74.

[16] Crook K, McLachlan G, Stevenson BJ, Porteous DJ. Plasmid DNA molecules complexed with cationic liposomes are protected from degradation by nucleases and shearing by aerosolisation. Gene Ther 1996 Sep;3(9):834-9.

[17] Goh SL, Murthy N, Xu M, Frechet JM. Cross-linked microparticles as carriers for the delivery of plasmid DNA for vaccine development. Bioconjug Chem 2004 May;15(3):467-74.

[18] Song YK, Liu F, Liu D. Enhanced gene expression in mouse lung by prolonging the retention time of intravenously injected plasmid DNA. Gene Ther 1998 Nov;5(11):1531-7.

[19] Thierry AR, Lunardi-Iskandar Y, Bryant JL, Rabinovich P, Gallo RC, Mahan LC. Systemic gene therapy: biodistribution and long-term expression of a transgene in mice. Proc Natl Acad Sci U S A 1995 Oct 10;92(21):9742-6.

[20] Rejman J, Oberle V, Zuhorn IS, Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. Biochem J 2004 Jan 1;377(Pt 1):159-69.

[21] van der Aa MA, Huth US, Hafele SY, et al. Cellular uptake of cationic polymer-DNA complexes via caveolae plays a pivotal role in gene transfection in COS-7 cells. Pharm Res 2007 Aug;24(8):1590-8.

[22] Mislick KA, Baldeschwieler JD. Evidence for the role of proteoglycans in cation-mediated gene transfer. Proc Natl Acad Sci U S A 1996 Oct 29;93(22):12349-54.

[23] Mir LM. Application of electroporation gene therapy: past, current, and future. Methods Mol Biol 2008;423:3-17.

[24] Andre F, Mir LM. DNA electrotransfer: its principles and an updated review of its therapeutic applications. Gene Ther 2004 Oct;11 Suppl 1:S33-S42.

[25] Daud AI, DeConti RC, Andrews S, et al. Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma. J Clin Oncol 2008 Dec 20;26(36):5896-903.

[26] Heller R, Jaroszeski MJ, Reintgen DS, et al. Treatment of cutaneous and subcutaneous tumors with electrochemotherapy using intralesional bleomycin. Cancer 1998 Jul 1;83(1):148-57.

[27] Dincer Z, Jones S, Haworth R. Preclinical safety assessment of a DNA vaccine using particle-mediated epidermal delivery in domestic pig, minipig and mouse. Exp Toxicol Pathol 2006 Jul;57(5-6):351-7.

[28] Ito K, Ito K, Shinohara N, Kato S. DNA immunization via intramuscular and intradermal routes using a gene gun provides different magnitudes and durations on immune response. Mol Immunol 2003 May;39(14):847-54.

[29] Jones S, Evans K, McElwaine-Johnn H, et al. DNA vaccination protects against an influenza challenge in a double-blind randomised placebo-controlled phase 1b clinical trial. Vaccine 2009 Apr 21;27(18):2506-12.

[30] Bahloul C, Taieb D, Diouani MF, et al. Field trials of a very potent rabies DNA vaccine which induced long lasting virus neutralizing antibodies and protection in dogs in experimental conditions. Vaccine 2006 Feb 20;24(8):1063-72.

[31] Tsai KC, Liao ZK, Yang SJ, et al. Differences in gene expression between sonoporation in tumor and in muscle. J Gene Med 2009 Jul 28.

[32] Taniyama Y, Tachibana K, Hiraoka K, et al. Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. Gene Ther 2002 Mar;9(6):372-80.

[33] Bao S, Thrall BD, Miller DL. Transfection of a reporter plasmid into cultured cells by sonoporation in vitro. Ultrasound Med Biol 1997;23(6):953-9.

[34] Bins AD, Jorritsma A, Wolkers MC, et al. A rapid and potent DNA vaccination strategy defined by in vivo monitoring of antigen expression. Nat Med 2005 Aug;11(8):899-904.

[35] Quaak SG, van den Berg JH, Toebes M, et al. GMP production of pDERMATT for vaccination against melanoma in a phase I clinical trial. Eur J Pharm Biopharm 2008 Oct;70(2):429-38.

[36] Trimble CL, Peng S, Kos F, et al. A phase I trial of a human papillomavirus DNA vaccine for HPV16+ cervical intraepithelial neoplasia 2/3. Clin Cancer Res 2009 Jan 1;15(1):361-7.

[37] Pavlenko M, Roos AK, Lundqvist A, et al. A phase I trial of DNA vaccination with a plasmid expressing prostate-specific antigen in patients with hormone-refractory prostate cancer. Br J Cancer 2004 Aug 16;91(4):688-94.

[38] Low L, Mander A, McCann K, et al. DNA vaccination with electroporation induces increased antibody responses in patients with prostate cancer. Hum Gene Ther 2009 Nov;20(11):1269-78.

[39] McNeel DG, Dunphy EJ, Davies JG, et al. Safety and immunological efficacy of a DNA vaccine encoding prostatic acid phosphatase in patients with stage D0 prostate cancer. J Clin Oncol 2009 Sep 1;27(25):4047-54.

[40] Triozzi PL, Aldrich W, Allen KO, Carlisle RR, LoBuglio AF, Conry RM. Phase I study of a plasmid DNA vaccine encoding MART-1 in patients with resected melanoma at risk for relapse. J Immunother (1997) 2005 Jul;28(4):382-8.

[41] Cassaday RD, Sondel PM, King DM, et al. A phase I study of immunization using particle-mediated epidermal delivery of genes for gp100 and GM-CSF into uninvolved skin of melanoma patients. Clin Cancer Res 2007 Jan 15;13(2 Pt 1):540-9.

[42] Rosenberg SA, Yang JC, Sherry RM, et al. Inability to immunize patients with metastatic melanoma using plasmid DNA encoding the gp100 melanoma-melanocyte antigen. Hum Gene Ther 2003 May 20;14(8):709-14.

[43] Gonzalez R, Hutchins L, Nemunaitis J, Atkins M, Schwarzenberger PO. Phase 2 trial of Allovectin-7 in advanced metastatic melanoma. Melanoma Res 2006 Dec;16(6):521-6.

[44] Stopeck AT, Jones A, Hersh EM, et al. Phase II study of direct intralesional gene transfer of allovectin-7, an HLA-B7/beta2-microglobulin DNA-liposome complex, in patients with metastatic melanoma. Clin Cancer Res 2001 Aug;7(8):2285-91.

[45] Hui KM, Ang PT, Huang L, Tay SK. Phase I study of immunotherapy of cutaneous metastases of human carcinoma using allogeneic and xenogeneic MHC DNA-liposome complexes. Gene Ther 1997 Aug;4(8):783-90.

[46] Isner JM, Pieczek A, Schainfeld R, et al. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. Lancet 1996 Aug 10;348(9024):370-4.

[47] Kusumanto YH, van Weel V, Mulder NH, et al. Treatment with intramuscular vascular endothelial growth factor gene compared with placebo for patients with diabetes mellitus and critical limb ischemia: a double-blind randomized trial. Hum Gene Ther 2006 Jun;17(6):683-91.

[48] Morishita R, Aoki M, Hashiya N, et al. Safety evaluation of clinical gene therapy using hepatocyte growth factor to treat peripheral arterial disease. Hypertension 2004 Aug;44(2):203-9.

[49] Powell RJ, Simons M, Mendelsohn FO, et al. Results of a double-blind, placebo-controlled study to assess the safety of intramuscular injection of hepatocyte growth factor plasmid to improve limb perfusion in patients with critical limb ischemia. Circulation 2008 Jul 1;118(1):58-65.

[50] Melman A, Bar-Chama N, McCullough A, Davies K, Christ G. hMaxi-K gene transfer in males with erectile dysfunction: results of the first human trial. Hum Gene Ther 2006 Dec;17(12):1165-76.

[51] Comerota AJ, Throm RC, Miller KA, et al. Naked plasmid DNA encoding fibroblast growth factor type 1 for the treatment of end-stage unreconstructible lower extremity ischemia: preliminary results of a phase I trial. J Vasc Surg 2002 May;35(5):930-6.

[52] Garver KA, LaPatra SE, Kurath G. Efficacy of an infectious hematopoietic necrosis (IHN) virus DNA vaccine in Chinook Oncorhynchus tshawytscha and sockeye O. nerka salmon. Dis Aquat Organ 2005 Apr 6;64(1):13-22.

[53] Thacker EL, Holtkamp DJ, Khan AS, Brown PA, Draghia-Akli R. Plasmid-mediated growth hormone-releasing hormone efficacy in reducing disease associated with Mycoplasma hyopneumoniae and porcine reproductive and respiratory syndrome virus infection. J Anim Sci 2006 Mar;84(3):733-42.

[54] Davis BS, Chang GJ, Cropp B, et al. West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses in vitro a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. J Virol 2001 May;75(9):4040-7.

[55] Bergman PJ, Camps-Palau MA, McKnight JA, et al. Development of a xenogeneic DNA vaccine program for canine malignant melanoma at the Animal Medical Center. Vaccine 2006 May 22;24(21):4582-5.

[56] Ledwith BJ, Manam S, Troilo PJ, et al. Plasmid DNA vaccines: investigation of integration into host cellular DNA following intramuscular injection in mice. Intervirology 2000;43(4-6):258-72.

[57] Cole J, Skopek TR. International Commission for Protection Against Environmental Mutagens and Carcinogens. Working paper no. 3. Somatic mutant frequency, mutation rates and mutational spectra in the human population in vivo. Mutat Res 1994 Jan;304(1):33-105.

[58] Pal R, Yu Q, Wang S, et al. Definitive toxicology and biodistribution study of a polyvalent DNA prime/protein boost human immunodeficiency virus type 1 (HIV-1) vaccine in rabbits. Vaccine 2006 Feb 20;24(8):1225-34.

[59] Manam S, Ledwith BJ, Barnum AB, et al. Plasmid DNA vaccines: tissue distribution and effects of DNA sequence, adjuvants and delivery method on integration into host DNA. Intervirology 2000;43(4-6):273-81.

[60] Martin T, Parker SE, Hedstrom R, et al. Plasmid DNA malaria vaccine: the potential for genomic integration after intramuscular injection. Hum Gene Ther 1999 Mar 20;10(5):759-68.

[61] Nichols WW, Ledwith BJ, Manam SV, Troilo PJ. Potential DNA vaccine integration into host cell genome. Ann N Y Acad Sci 1995 Nov 27;772:30-9.

[62] Wang Z, Troilo PJ, Wang X, et al. Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation. Gene Ther 2004 Apr;11(8):711-21.

[63] Sheets RL, Stein J, Manetz TS, et al. Biodistribution of DNA plasmid vaccines against HIV-1, Ebola, Severe Acute Respiratory Syndrome, or West Nile virus is similar, without integration, despite differing plasmid backbones or gene inserts. Toxicol Sci 2006 Jun;91(2):610-9.

[64] Weaver D, Boubnov N, Wills Z, Hall K, Staunton J. V(D)J recombination: double-strand break repair gene products used in the joining mechanism. Ann N Y Acad Sci 1995 Sep 29;764:99-111.

[65] Wahls WP, Wallace LJ, Moore PD. Hypervariable minisatellite DNA is a hotspot for homologous recombination in human cells. Cell 1990 Jan 12;60(1):95-103.

[66] Rudiger NS, Gregersen N, Kielland-Brandt MC. One short well conserved region of Alu-sequences is involved in human gene rearrangements and has homology with prokaryotic chi. Nucleic Acids Res 1995 Jan 25;23(2):256-60.

[67] Dean DA. Import of plasmid DNA into the nucleus is sequence specific. Exp Cell Res 1997 Feb 1;230(2):293-302.

[68] Li S, MacLaughlin FC, Fewell JG, et al. Muscle-specific enhancement of gene expression by incorporation of SV40 enhancer in the expression plasmid. Gene Ther 2001 Mar;8(6):494-7.

[69] Young JL, Benoit JN, Dean DA. Effect of a DNA nuclear targeting sequence on gene transfer and expression of plasmids in the intact vasculature. Gene Ther 2003 Aug;10(17):1465-70.

[70] Goncalves C, Ardourel MY, Decoville M, et al. An optimized extended DNA kappa B site that enhances plasmid DNA nuclear import and gene expression. J Gene Med 2009 May;11(5):401-11.

[71] Mazda O. Improvement of nonviral gene therapy by Epstein-Barr virus (EBV)-based plasmid vectors. Curr Gene Ther 2002 Sep;2(3):379-92.

[72] Ivics Z, Izsvak Z. Transposons for gene therapy! Curr Gene Ther 2006 Oct;6(5):593-607.

[73] Wilson MH, Coates CJ, George AL, Jr. PiggyBac transposon-mediated gene transfer in human cells. Mol Ther 2007 Jan;15(1):139-45.

[74] Mates L, Chuah MK, Belay E, et al. Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. Nat Genet 2009 Jun;41(6):753-61.

[75] Peng PD, Cohen CJ, Yang S, et al. Efficient nonviral Sleeping Beauty transposon-based TCR gene transfer to peripheral blood lymphocytes confers antigen-specific antitumor reactivity. Gene Ther 2009 Aug;16(8):1042-9.

[76] van Gaal EV, Hennink WE, Crommelin DJ, Mastrobattista E. Plasmid engineering for controlled and sustained gene expression for nonviral gene therapy. Pharm Res 2006 Jun;23(6):1053-74.

[77] Van Craenenbroeck K, Vanhoenacker P, Haegeman G. Episomal vectors for gene expression in mammalian cells. Eur J Biochem 2000 Sep;267(18):5665-78.

[78] Ohlschlager P, Pes M, Osen W, et al. An improved rearranged Human Papillomavirus Type 16 E7 DNA vaccine candidate (HPV-16 E7SH) induces an E7 wildtype-specific T cell response. Vaccine 2006 Apr 5;24(15):2880-93.

[79] Mir LM, Bureau MF, Gehl J, et al. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. Proc Natl Acad Sci U S A 1999 Apr 13;96(8):4262-7.

[80] Itaka K, Osada K, Morii K, Kim P, Yun SH, Kataoka K. Polyplex nanomicelle promotes hydrodynamic gene introduction to skeletal muscle. J Control Release 2010 Jan 3.

[81] Chang CW, Choi D, Kim WJ, et al. Non-ionic amphiphilic biodegradable PEG-PLGA-PEG copolymer enhances gene delivery efficiency in rat skeletal muscle. J Control Release 2007 Apr 2;118(2):245-53.

[82] Roos AK, Eriksson F, Timmons JA, et al. Skin electroporation: effects on transgene expression, DNA persistence and local tissue environment. PLoS One 2009;4(9):e7226.

[83] Luckay A, Sidhu MK, Kjeken R, et al. Effect of plasmid DNA vaccine design and in vivo electroporation on the resulting vaccine-specific immune responses in rhesus macaques. J Virol 2007 May;81(10):5257-69.

[84] Vilalta A, Mahajan RK, Hartikka J, et al. I. Poloxamer-formulated plasmid DNA-based human cytomegalovirus vaccine: evaluation of plasmid DNA biodistribution/persistence and integration. Hum Gene Ther 2005 Oct;16(10):1143-50.

[85] Vilalta A, Mahajan RK, Hartikka J, et al. II. Cationic lipid-formulated plasmid DNA-based Bacillus anthracis vaccine: evaluation of plasmid DNA persistence and integration potential. Hum Gene Ther 2005 Oct;16(10):1151-6.

[86] Gravier R, Dory D, Laurentie M, Bougeard S, Cariolet R, Jestin A. In vivo tissue distribution and kinetics of a pseudorabies virus plasmid DNA vaccine after intramuscular injection in swine. Vaccine 2007 Sep 28;25(39-40):6930-8.

[87] Liu FY, Guo RM, Zheng L, et al. Safety evaluation of a canine hepatitis DNA vaccine. Vaccine 2008 Dec 9;26(52):6925-8.

[88] Melman A, Bar-Chama N, McCullough A, Davies K, Christ G. Plasmid-based gene transfer for treatment of erectile dysfunction and overactive bladder: results of a phase I trial. Isr Med Assoc J 2007 Mar;9(3):143-6.

[89] Gao YS, Huang TH, Wang D, Xie QD, Kang XJ. In Vivo Study on Vertical Transmission of the HIV-1 gag Gene via Mouse Oocytes. Curr HIV Res 2009 Sep;7(5):562-8.

[90] Nabel EG, Gordon D, Yang ZY, et al. Gene transfer in vivo with DNA-liposome complexes: lack of autoimmunity and gonadal localization. Hum Gene Ther 1992 Dec;3(6):649-56.

[91] Parrington J, Coward K, Hibbitt O, et al. In vivo gene transfer into the testis by electroporation and viral infection--a novel way to study testis and sperm function. Soc Reprod Fertil Suppl 2007;65:469-74.

[92] Hibbitt O, Coward K, Kubota H, et al. In vivo gene transfer by electroporation allows expression of a fluorescent transgene in hamster testis and epididymal sperm and has no adverse effects upon testicular integrity or sperm quality. Biol Reprod 2006 Jan;74(1):95-101.

[93] Schubbert R, Renz D, Schmitz B, Doerfler W. Foreign (M13) DNA ingested by mice reaches peripheral leukocytes, spleen, and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA. Proc Natl Acad Sci U S A 1997 Feb 4;94(3):961-6.

[94] Hohlweg U, Doerfler W. On the fate of plant or other foreign genes upon the uptake in food or after intramuscular injection in mice. Mol Genet Genomics 2001 Apr;265(2):225-33.

[95] Schubbert R, Hohlweg U, Renz D, Doerfler W. On the fate of orally ingested foreign DNA in mice: chromosomal association and placental transmission to the fetus. Mol Gen Genet 1998 Oct;259(6):569-76.

[96] Orsag P, Kvardova V, Raska M, Miller AD, Ledvina M, Turanek J. Quantitative real-time PCR study on persistence of pDNA vaccine pVax-Hsp60 TM814 in beef muscles. Genet Vaccines Ther 2008;6:11.

[97] Gaytant MA, Galama JM, Semmekrot BA, et al. The incidence of congenital cytomegalovirus infections in The Netherlands. J Med Virol 2005 May;76(1):71-5.

[98] Wu Y, Liu G, Carstens EB. Replication, integration, and packaging of plasmid DNA following cotransfection with baculovirus viral DNA. J Virol 1999 Jul;73(7):5473-80.

[99] Evans DH, Stuart D, McFadden G. High levels of genetic recombination among cotransfected plasmid DNAs in poxvirus-infected mammalian cells. J Virol 1988 Feb;62(2):367-75.

[100] Comerota AJ, Throm RC, Miller KA, et al. Naked plasmid DNA encoding fibroblast growth factor type 1 for the treatment of end-stage unreconstructible lower extremity ischemia: preliminary results of a phase I trial. J Vasc Surg 2002 May;35(5):930-6.

[101] Wolff JA, Ludtke JJ, Acsadi G, Williams P, Jani A. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. Hum Mol Genet 1992 Sep;1(6):363-9.

[102] Zhang HY, Sun SH, Guo YJ, et al. Tissue distribution of a plasmid DNA containing epitopes of foot-and-mouth disease virus in mice. Vaccine 2005 Dec 1;23(48-49):5632-40.

[103] Ramirez K, Barry EM, Ulmer J, et al. Preclinical safety and biodistribution of Sindbis virus measles DNA vaccines administered as a single dose or followed by live attenuated measles vaccine in a heterologous prime-boost regimen. Hum Gene Ther 2008 May;19(5):522-31.

[104] Winter V, Freund D. Demonstration of ribonuclease and desoxyribonuclease in human skin by means of fluorescent antibodies. J Invest Dermatol 1969 Apr;52(4):344-7.

[105] Lorenz MG, Wackernagel W. Bacterial gene transfer by natural genetic transformation in the environment. Microbiol Rev 1994 Sep;58(3):563-602.

[106] Martin S, Failla AV, Spori U, Cremer C, Pombo A. Measuring the size of biological nanostructures with spatially modulated illumination microscopy. Mol Biol Cell 2004 May;15(5):2449-55.

[107] Dalboge H, Madsen B, Jorgensen KD, Carlsen S. Assessment of risks in connection with use of a recombinant E. coli strain for production of human growth hormone. Dan Med Bull 1988 Feb;35(1):84-91.

[108] Seidman CE, Struhl K. Introduction of plasmid DNA into cells. Curr Protoc Protein Sci 2001 May;Appendix 4:4D.

[109] Hakenbeck R, Grebe T, Zahner D, Stock JB. beta-lactam resistance in Streptococcus pneumoniae: penicillin-binding proteins and non-penicillin-binding proteins. Mol Microbiol 1999 Aug;33(4):673-8.

[110] Smith HO, Gwinn ML, Salzberg SL. DNA uptake signal sequences in naturally transformable bacteria. Res Microbiol 1999 Nov;150(9-10):603-16.

[111] Treangen TJ, Ambur OH, Tonjum T, Rocha EP. The impact of the neisserial DNA uptake sequences on genome evolution and stability. Genome Biol 2008;9(3):R60.

[112] COMMITTEE FOR MEDICINAL PRODUCTS FOR VETERINARY USEAND COMMITTEE FOR MEDICINAL PRODUCTS FOR HUMAN USE. Presence of the antibiotic resistance marker gene nptII in GM plants for food and feed uses. 2007 Feb 22.

[113] Williams JA, Carnes AE, Hodgson CP. Plasmid DNA vaccine vector design: impact on efficacy, safety and upstream production. Biotechnol Adv 2009 Jul;27(4):353-70.

[114] Petrocheilou V, Richmond MH. Absence of plasmid or Escherichia coli K-12 infection among laboratory personnel engaged in R-Plasmid research. Gene 1977;2(5-6):323-7.

[115] Inoue Y. Spontaneous loss of antibiotic-resistant plasmids transferred to Escherichia coli in experimental chronic bladder infection. Int J Urol 1997 May;4(3):285-8.

[116] Soubrier F, Cameron B, Manse B, et al. pCOR: a new design of plasmid vectors for nonviral gene therapy. Gene Ther 1999 Aug;6(8):1482-8.

[117] Vidal L, Pinsach J, Striedner G, Caminal G, Ferrer P. Development of an antibiotic-free plasmid selection system based on glycine auxotrophy for recombinant protein overproduction in Escherichia coli. J Biotechnol 2008 Mar 20;134(1-2):127-36.

[118] Dong WR, Xiang LX, Shao JZ. A Novel Antibiotic-free Plasmid Selection System Based on Complementation of Host Auxotrophy in NAD De Novo Synthesis Pathway. Appl Environ Microbiol 2010 Jan 29.

[119] Cranenburgh RM, Hanak JA, Williams SG, Sherratt DJ. Escherichia coli strains that allow antibiotic-free plasmid selection and maintenance by repressor titration. Nucleic Acids Res 2001 Mar 1;29(5):E26.

[120] Eastman EM, Durland RH. Manufacturing and quality control of plasmid-based gene expression systems. Adv Drug Deliv Rev 1998 Mar 2;30(1-3):33-48.

[121] Luke J, Carnes AE, Hodgson CP, Williams JA. Improved antibiotic-free DNA vaccine vectors utilizing a novel RNA based plasmid selection system. Vaccine 2009 Oct 30;27(46):6454-9.

[122] Mayrhofer P, Schleef M, Jechlinger W. Use of minicircle plasmids for gene therapy. Methods Mol Biol 2009;542:87-104.

[123] Olasz F, Farkas T, Kiss J, Arini A, Arber W. Terminal inverted repeats of insertion sequence IS30 serve as targets for transposition. J Bacteriol 1997 Dec;179(23):7551-8.

[124] Dower WJ, Miller JF, Ragsdale CW. High efficiency transformation of E. coli by high voltage electroporation. Nucleic Acids Res 1988 Jul 11;16(13):6127-45.

Appendix I: Environmental risk of DNA containing nanoparticle formulations

DNA delivery by synthetic carriers might potentially increase the environmental risk of naked DNA. Cationic carriers are widely used to increase transfection of eukaryotic cells. These systems protect DNA from endonuclease-mediated degradation and improve the interaction with negatively charged cells. Synthetic carriers might also increase transformation of bacteria, since bacteria also possess a negatively charged cell wall. To our knowledge, this risk has never been assessed in literature. In an attempt to fill this gap in the literature, we performed a pilot study in order to determine the transforming ability of a non-viral carrier system shed into the environment. For that an *in vitro* experiment was performed in which *E.coli* DH5α was incubated with different concentrations of positively charged DNA/liposome complexes, neutrally charged DNA/liposome complexes or naked pDNA solution (using a kanamycin resistance pDNA construct, pVAX1). Upon incubation, *E.coli* was plated on kanamycin containing agar plates to determine the transforming ability of complexes and naked DNA under the "worst case" situation of selection pressure (see Table VI). Colony forming units were absent or present at extremely low number, suggesting that the transforming ability of both naked DNA and DNA/liposomes complexes shed into the environment is extremely low. In addition, competent *E. coli DH5α* was transformed with DNA/liposome complexes and naked DNA by a standard heat shock protocol. Formulation of pDNA with a liposome decrease the amount of colony forming units compared to naked DNA. Probably, dissociation of the pDNA from the liposomal carrier when reached the cytoplasm or cytotoxicity is limiting transformation. These preliminary observations obtained from one model bacterium, suggest that formulation of DNA with a synthetic carrier does not result in an increased bacterial uptake compared to naked DNA in solution, if shed into the environment.

Table VI: number of colony forming units (cfu) of E.coli DH5α incubated or heat shocked with naked DNA or DNA/liposomes complexes (lipoplex) at positive or neutral charge. Values represent the mean of 3 independent experiments (with standard deviation).

Exposure	Formulation	Amount DNA	cfu	Exposure	Formulation	Amount DNA	cfu
Incubation	Naked DNA	1 µg	0 (0)	Heat shock	Naked DNA	1 µg	>10.000
		100 ng	0 (0)			100 ng	>1.000
		10 ng	0 (0)			10 ng	470 (217)
	Cationic lipoplex	1 µg	1 (1)		Cationic lipoplex	1 µg	0.3 (0.6)
		100 ng	1 (1)			100 ng	0 (0)
		10 ng	0 (0)			10 ng	0 (0)
	Neutral lipoplex	1 µg	0 (0)		Neutral lipoplex	1 µg	>10.000
		100 ng	0 (0)			100 ng	>1.000
		10 ng	0 (0)			10 ng	150 (56)

Materials and Methods

Materials

The pVAX:Luc-NP plasmid[34] 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 pDNA backbone (Invitrogen, Carlsbad, USA) was used. Plasmid was expressed and amplified in *E.coli* DH5α and was purified by Endofree™ QIAGEN® Mega-kit (QIAGEN®, Hilden, Germany). 1,2-dioleoyl-oxypropyl-3-trimethyl-ammonium chloride (DOTAP) was obtained from Avanti Polar lipids (Alabster, AL, USA). Dioleoylphosphatidyl-ethanolamide (DOPE) and distearoylphosphatidylethanolamine-polyethyleneglycol 2000 (DSPE-PEG) were a kind gift from Lipoïd GmbH (Ludwigshafen, Germany).

Liposome preparation

Cationic liposomes, composed of DOTAP-DOPE, were prepared in a 1:1 molar ratio. For neutral, PEGylated liposomes, DOPE was replaced by DSPE-PEG at different concentrations to keep the total molarity of lipids constant. Lipid mixtures were dissolved in chloroform/methanol (1:1 v/v) and mixed in a round-bottomed flask. Organic solvents were evaporated at 40 ºC using a vacuum evaporator and the obtained lipid films were purged with nitrogen for 30 min. Lipid films were rehydrated in 20 mM HEPES, pH 7.4, 5% glucose, to give a final lipid concentration of 7 mM. The resuspended lipids were extruded 8 times through two stacked polycarbonate membranes (Poretics, Livermore, USA, 200 and 100 nm) to obtain small unilamellar vesicles of 100 nm.

Lipoplex preparation

DNA/liposome complexes (lipoplexes) were prepared by mixing an equal volume of pDNA solution with cationic liposomes or neutral liposomes containing DSPE-PEG. Lipoplexes were prepared in 20 mM HEPES pH 7.4, 5% glucose buffer. The charge ratio between cationic nitrogen residues in DOTAP and anionic phosphate groups in the DNA was set on 5. Control naked DNA was diluted to the same concentration in the same buffer.

In vitro incubation

1.10^6 colony forming units *E.coli* DH5α were dispersed in 100µl Luria broth (LB) medium. The obtained cultures were incubated for 45min at 37°C with 5µl formulation corresponding with 1µg, 100ng or 10ng pVAX:LucNP plasmid. Upon incubation, *E.coli* cultures were plated onto kanamycin (100µg/ml) containing LB agar plates and incubated overnight at 37°C to determine the number of transformed bacteria.

Heat shock transformation

E.coli DH5α were made competent with $CaCl_2$ and $2.5.10^7$ colony forming units of these competent cells in 50 µl were subsequently transformed with 5µl formulation corresponding with 1µg, 100ng or 10ng pVAX:LucNP plasmid using a standard heat shock protocol.

Appendix II: Environmental risk of DNA tattoo vaccination

Tattooing is a dermal delivery technique that might lead to increased levels of environmental risk, since the tattoo needles, that oscillate with a frequency of 100Hz, can easily spread out the DNA over a larger area of skin or into the environment (possible via aerosol formation). Additionally, shed DNA can be taken up by present skin flora. For that, we consider this administration technique as a "worst case" scenario in terms of environmental risk.

To evaluate the environmental risk in a clinical situation, the number of transformed skin bacteria in melanoma patients that were tattoo vaccinated with a naked DNA vaccine (pDERMATT[35]) was determined. The pDERMATT constructs encodes a melanoma antigen inserted in a kanamycin resistance pVAX1 backbone. Upon tattoo application of the vaccine, the tattooed skin area was wiped by a cotton swab. This sample was subsequently plated on agar plates with or without kanamycin to determine the presence of transformed and non-transformed skin flora. In none of the patient samples or gloves of the administrator, transformed kanamcyin resistance bacteria could be detected (see Table VII). However, we should address that the amount of colony forming units on the plates without antibiotics was also relatively low. Although these data should be interpreted with caution, since the number of colonies was low and only one growth condition was tested, these preliminary data suggest that the bacterial transformation upon dermal tattooing is not markedly increased.

Table VII: number of colony forming units (cfu) in skin swabs from patients tattooed with a kanymycine resistance DNA vaccine (pDERMATT[35]) and swabs from the gloves of the administrator.

Patient identifier	Tattoo number	Location	Antibiotic on plate	cfu
1 (female)	2	Gloves	Kanamycin	0
		Tattooed skin	None	0
		Tattooed skin	Kanamycin	0
		Control skin	None	10
		Control skin	Kanamycin	0
1 (female)	3	Gloves	Kanamycin	0
		Tattooed skin	None	0
		Tattooed skin	Kanamycin	0
		Control skin	None	44
		Control skin	Kanamycin	0
2 (male)	1	Gloves	Kanamycin	0
		Tattooed skin	None	44
		Tattooed skin	Kanamycin	0
		Control skin	None	8
		Control skin	Kanamycin	0
2 (male)	2	Gloves	Kanamycin	0
		Tattooed skin	None	516
		Tattooed skin	Kanamycin	0
		Control skin	None	104
		Control skin	Kanamycin	0
2 (male)	3	Gloves	Kanamycin	0
		Tattooed skin	None	16
		Tattooed skin	Kanamycin	0
		Control skin	None	4
		Control skin	Kanamycin	0
1 (female)	4	Gloves	Kanamycin	0
		Tattooed skin	None	0
		Tattooed skin	Kanamycin	0
		Control skin	None	536
		Control skin	Kanamycin	8
1 (female)	5	Gloves	Kanamycin	0
		Tattooed skin	None	8
		Tattooed skin	Kanamycin	0
		Control skin	None	40
		Control skin	Kanamycin	0

Materials and Methods

DNA vaccine

The melanoma DNA vaccine, encoding MART-1 and tetanus toxin fragment-c inserted in the pVAX1 plasmid backbone (Invitrogen, Carlsbad, USA) was produced in house as described before[35].

Tattooing administration and sampling of patients

Stage III and IV melanoma patients were tattoo vaccinated as a part of a clinical trial. Patients received a dose of 1 mg pDERMATT (in 200µl 10% w/v sucrose), tattooed over an area of 8 cm^2 for a period of 5 minutes. The vaccine was administered using a Permanent Make Up (PMU) ® tattoo machine (kindly provided by MT Derm GmbH, Berlin, Germany). For all tattoos, 9-needle cartridges at an oscillating frequency of 100 Hz were used. The needle depth was adjusted to 1.5 mm.

Ten minutes after administration, tattooed areas were wiped with a sterile cotton swab, moisturized with PBS. The cotton swabs were transferred into 400µl of PBS, incubated for 30 minutes at room temperature and plated onto LB agar plates containing kanamycin (100µg/ml) or no antibiotics. After two days of incubation at 37°C, the number of colonies was determined.