Microparticular and Nanoparticular DNA Delivery Systems as Adjuvants for DNA Immunization

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Meinen Eltern

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CHAPTER 1

INTRODUCTION

DNA VACCINES

Immunization is regarded as one of the most significant successes in medical development of the past two hundred years. When Edward Jenner, in 1786, performed the first vaccination on an eight year old boy, he deliberately applied the first live attenuated vaccine. Since then other remarkable developments have resulted in successful vaccines against small pox, polio, measles and rubella. In total, twenty-six infectious diseases are preventable by vaccination, leading to an estimated 10 - 15 years longer average lifetime of men in the 20th century.

Live attenuated vaccines still represent the most successful vaccines. They often induce a life long protection by an active infection of the attenuated pathogen resulting in the development of an effective immunologic memory. However, infectious diseases, such as malaria, tuberculosis and especially HIV, cannot be controlled using these vaccines. This is either due to by the possibility of restored pathogen virulence, the difficulty to vaccinate immunodefficient patients or the frequent mutation of some pathogens, thereby escaping anterior immunizations.

Developments of the past 30 years have led to vaccines, such as subunit vaccines (Fluad®, Chiron Behring), recombinant protein vaccines (Engerix-B®, GlaxoSmithKline) and protein-polysaccharide conjugate based vaccines (Meningitec®, Wyeth). Despite the new potentials arising from the broader possibilities to immunize safely compared to live vaccines, these vaccines are less immunogenic. Especially the reduced capacity to induce cell mediated immune responses of the protein and peptide antigens could not be completely overcome by adjuvant systems. Therefore, the challenges remain to develop potent, but safe vaccines against infectious diseases, cancer and autoimmune diseases, whereas the latter are mostly dependent on a cytotoxic T-cell response.

The potential of using DNA as a vaccine was discovered by gene therapists. They detected immune responses against the proteins that had been genetically

delivered in the form of plasmid DNA. This knowledge was only used for the purpose of DNA vaccination in the early 90´s. Several studies in mice then revealed protection against pathogen challenge, mainly influenza.

The mechanisms of the induction of cellular and humoral immune response are summarized in Figure 1.

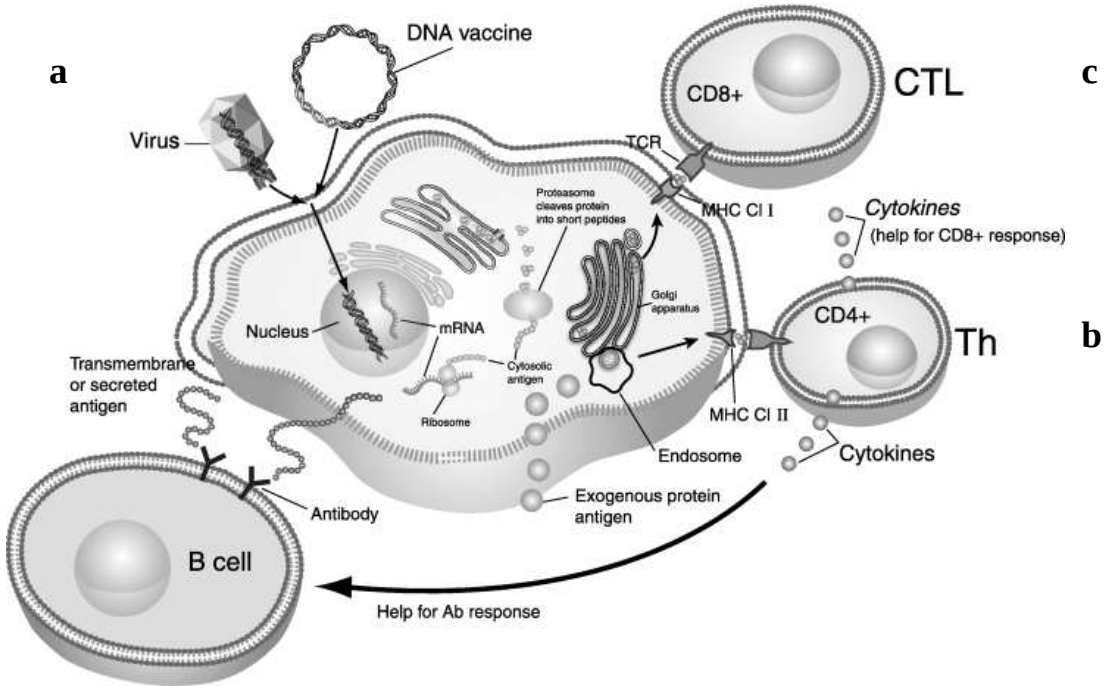

Fig.1: *Mechanism of antigen-specific humoral and cellular immune response [1]. (a) Recognition of exogenous antigen by APC and activation of a humoral immune response. (b) T-helper cell activation by antigen presentation via MHC II molecules of the transfected APC. (c) Activation of cytotoxic T- lymphocytes (CTL) by the presentation of foreign peptides synthesized and processed by the transfected cell.*

The levels of immune response generated by the different immunization strategies are summarized in Table 1. The similarity of the immune response obtained by DNA -and live attenuated vaccines, as well as the increased safety of DNA vaccines are highlighted in the Table 1.

Immune response		**DNA vaccine**	**Live attenuated vaccine**	**Protein/ Subunit/ Inactivated**
Humoral	B cells	+++	+++	+++
	$CD4^+$	++	+/- Th1	+/- Th1
Cellular	$CD8^+$	++	+++	-
Antigen presentation		MHC I / II	MHC I / II	MHC II
	Humoral	+++	+++	+++
	Cellular	++	+++	+/-
Manufacturing	Ease of development	++++	+	++
	Costs	++	+	+
	Transport /storage	++	+	+++
Safety		+++	++	++++

Table 1: *Quality of immune responses obtained with DNA vaccines, live attenuated vaccines and protein / subunits or inactivated vaccines.*

The great advantage of DNA vaccines is their ability to induce a humoral as well as a cellular type of immune reaction.

The encoded antigenic protein can be either processed via the intrinsic presentation pathway and presented by MHC I (major histocompatibility) molecules. Alternatively the antigen can be presented by MHC II molecules, which are specific for antigen presenting cells and some endothelial cells. This provides the opportunity of a vaccine corresponding better to a live vaccine type of response without the dangers associated with an infection of attenuated bacteria or viruses. The ability of DNA vaccines to generate potent cytotoxic T-

lymphocyte (CTL) responses is a major advantage. The activation of CTL depends on in-situ protein synthesis and subsequent presentation via the MHC I molecule. The in-situ synthesis results in posttranslational modifications, such as glycosylation, proteolytic processing, as well as lipid conjugation. Thus, the "naïve" form of the antigenic protein is produced and leads to a better recognition of the upcoming antigen after pathogen infection. The generation of T-cell responses by genetic vaccines was identified as a promising strategy to act against intracellular bacteria and parasites, as well as viral infections and cancer.

DNA immunizations were performed by either direct intra-muscular injection of naked DNA or by the use of a gene gun, with DNA coated gold microprojectiles (Fig.2) [2-6].

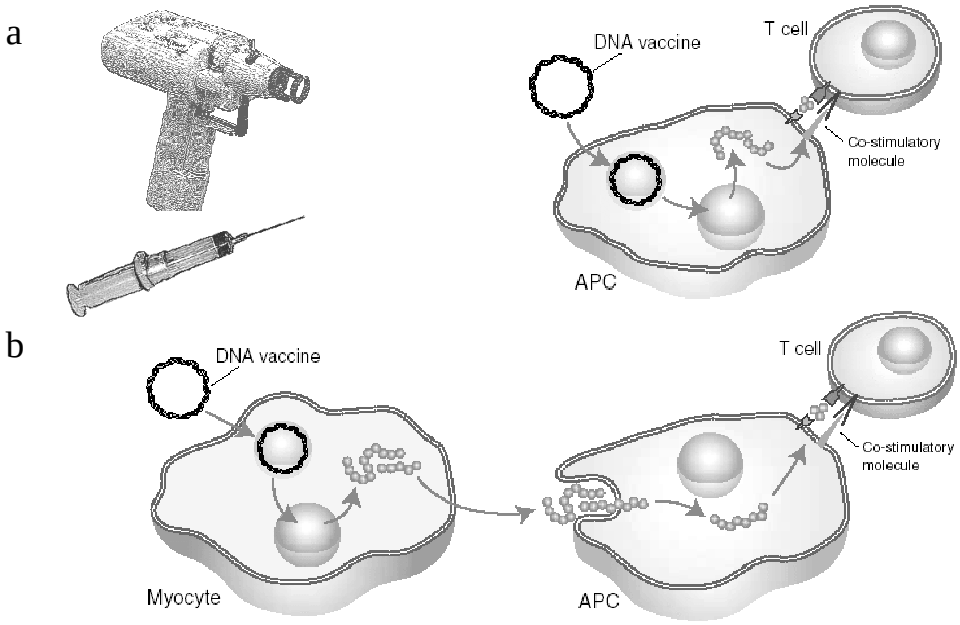

Fig.2: *Application and T-cell activation mechanisms of DNA vaccines [1]. (a) Gene gun application or injection of naked plasmid DNA into the skin or the muscle. Direct transfection of antigen presenting cells (APC), either dendritic cells or dendritic cells of the skin, Langerhans cells, and presentation to T-cells. (b) Transfection of myocytes and 'cross priming', the transfer of the antigen to a APC, and further activation of T-cells.*

It has not been clear for some time by which mechanism DNA immunization occurred.

The two possibilities for the induction of a CTL response consist of either direct DNA uptake into antigen presenting cells and the expression of the antigen. Alternatively, the protein synthesis occurs in non-antigen presenting cells with subsequent uptake into antigen presenting cells, referred to as ‘cross priming’ [7-9]. A third pathway comprising of the simple transfection of muscle cells could be ruled out. This mechanism would not induce humoral, antibody based reactions, because of the lack of MHC II presentation and because the lack of co-stimulatory molecules. The MHC II antigen presentation is restricted to antigen presenting cells and some endothelial cells.

In vitro studies have demonstrated the difficulty of transfecting antigen presenting cells and other phagocytes [10,11]. Still, Denis-Mize et al. demonstrated gene expression in dendritic cells by reverse transcriptase-PCR and by measuring the activation of an epitope-specific T-cell hybridoma by Il-2 expression [12]. This mechanism is of great importance as antigen presenting dendritic cells have the ability to prime naïve T-lymphocytes, resulting in significantly stronger T-cell responses [13]. Moreover, dendritic cells can directly activate CTL by the MHC I antigen presentation of phagocytosed apoptotic bodies [14].However, this mechanism is not fully understood and will have to be elucidated to effectively use plasmid DNA for vaccination. A protective immunization of small animal models, as well as some non-human primates has shown promising results.

Human clinical trials, including several against HIV and cancer have been initiated. An example of current human clinical trials is given in Table 2. The results of completed trials, however, could not confirm the effectiveness of DNA vaccines unless very high DNA doses were used [15,16]. Viral gene delivery has been used most frequently for gene therapy and has shown promising effects for DNA vaccination, as well.

Vaccine/ Condition	Clinical Phase	Plasmid	Dose	Administration
HIV / healthy volunteers	**Phase I** NIAID Study: HVTN048	EP HIV-1090 21 specific CTL epitopes	3 x	i.m. injection + PVP protects / facilitates DNA uptake
	Phase I NIAID Study: AVEG 031	APL 400-047 + Bupivacaine HCL	0.1 mg 0.3 mg 1 mg 3 mg	i.m. by needle or Biojector 2000 Needle-Free Jet Injection
	Phase I NIAID Study: HVTN 044	VRC-HIVDNA009-00-VP + IL-2/ Ig DNA adjuvant	4 x	needle-free i.m. injection DNA encoding IL-2 fused to the Fc portion of IgG for enhanced stability.
	Phase I NIAID Study: N01-AI05394	HIV-1 DNA vaccine with protein vaccine boost	3 x DNA 2 x protein	Polyvalent HIV-1 DNA plasmid prime/env protein boost vaccine
Melanoma Neoplasm Metastasis	**Phase I** NCI Study: 980086; 98-C-0086	gp100 DNA	4 x	+ IL-2
Leukemia, Chronic	**Phase I/II** M.D. Anderson Cancer Center Study: DM99-412	plasmid vector and DNA fragments		containing the sequence of their own immunoglobulin gene
Ebola / healthy volunteers	**Phase I** Vical / NIAID	VRC-EBODNA012-00-VP		

Table 2: *Examples of current clinical trials of DNA vaccines consisting predominantly of Phase I trials for immunization against HIV and cancer. The adjuvants used are IL-2, polyvinylpyrrolidone (PVP) and bupivacaine. The DNA is applied via i.m. injection or a Biojector®. Gene delivery via bacteria, viruses, and ex vivo transfection of cells were not analysed.*

However, viral delivery systems have led to fatal adverse immune reactions in a patient [17]. Therefore, we will focus on non viral adjuvants and delivery systems. Many other trials were conducted using viruses, which have not been found to be extensively effective [18].

Despite the disappointing results in humans until now, great successes in small animals and the theoretical possibilities, arising from the use of DNA vaccines legitimate further research and developments.

Advantages	Drawbacks
• Possibility to immunize against obligate intracellular bacteria such as Mycobacterium tuberculosis and Listeria m.	• Potential integration of the plasmid into host genome leading to insertional mutagenesis
• Supports CTL priming despite deficient T helper cells	• Induction of autoimmune responses (e.g. pathogenic anti-DNA antibodies)
• Antigens are equal to the antigenic proteins of a viral infection, due to post-translational modifications	• Effects of long-term expression unknown
• Plasmid are easily manufactured in large amounts	• Concept restricted to protein antigens
• DNA is more stable than proteins	• Induction of immunologic tolerance
• Fast adaptation of DNA vaccines is possible	
• Mixtures of plasmids encoding for multiple protein fragments are possible	
• Only the protein of interest is expressed.	
• No immune reaction against naked DNA or synthetic vectors.	
• Antigen does not have to be a pathogen surface characteristic for CTL response.	

Table 3: *Advantages and drawbacks of DNA vaccines.*

Therefore, DNA vaccines have to be improved. This is achieved using diverse possibilities such as genetic adjuvants, immuno-stimulatory agents encoded by the plasmid vector or the development of molecular adjuvants, such as cytokines.
Another promising tool to improve the immune response of DNA vaccines is the development of delivery systems that enhance the efficiency of gene delivery and provide a targeting of antigen presenting cells.

VACCINE ADJUVANTS

Adjuvants, e.g. substances that can enhance an immune response without being immunognic themselves, have been used since the early 1920 to improve vaccine efficacy [19,20]. Adjuvants demonstrate several properties. They

- increase the immune response of weakly immunogenic antigens
- decrease the dose necessary for successful immunization and reduce the number of boosts needed
- prolong the duration and speed-up the onset of the immune response
- modulate the immune response inducing different antibody isotypes or inducing mucosal immunity
- stimulate cytotoxic T lymphocytes
- facilitate the immunization with combined vaccines
- allow the immunization of elderly

The immune reaction induced by simple injection of plasmid DNA cannot achieve a sufficient immune response for protection against pathogenic challenge. Therefore, very early in the development of DNA vaccines the co-application of adjuvants was investigated.

Non-Particulate Adjuvants

The first, in the beginning undeliberately applied adjuvant of DNA vaccination were CpG (cytidine–phosphate–guanosine) dinucleotide motives of the procaryontic genetic material. CpG motives can be allocated within the group of non-particulate, soluble, adjuvants, in contrast to particulate adjuvants [27]. These sequences are sur-represented in procaryontic cells and occur four to five times more frequently than in eucaryontic cells. Thus, eucaryontic immune systems have evolved to recognize these sequences as danger signals of bacterial infections. Hence, the simple injection of plasmid DNA generated in bacteria, resulted in the activation of the immune system, namely the innate, unspecific type, by CpG-binding to the toll-like receptor (TLR) 9 [21,22]. The toll-like receptor family represent components that recognize evolutionarily conserved pathogen patterns. There are currently 10 known TLRs.

CpG motives further play an important role in the T helper cell pathway. They have been found to induce activation of the T helper cell 1 (T_H1) pathway on the disfavor of a T_H2 pathway, by up-regulating cytokines such as Il-12 [25,26]. T_H1 cells induce a CTL immune reaction, whereas T_H2 cells activate a humoral immune response.

Another adjuvant danger signal arising from bacterial genetic material is the rate of methylation of the nucleotides. Bacterial nucleotides are not methylated compared to a 75 % methylation to 5-methylcytosine in eucaryontic cells [23,24]. These differences in DNA composition result in relatively high immune responses of injected naked DNA.

Other non-particulate adjuvants are mainly immuno-modulators, such as cytokines (Il-2 and Il-12 up-regulate the T_H1 pathway [26,28]; Il-4 up-regulate the T_H2 pathway) and isolated substances from LPS (lipopolysaccharide), especially lipid A and MPL (monophosphoryl lipid A) which induce strong T_H1 responses, for example in hyposensibilizing injections (Pollinex® Quattro,

Bencard) [29-31]. Saponins, natural glycosides, are used in micellar preparations, such as Quil A in veterinary vaccines. These boost the T_H1, as well as the T_H2 pathway [32].

Particulate Adjuvants

An early in the development in genetic vaccines used DNA coated on gold microprojectiles which were propelled into the skin of mice by the so-called 'gene gun'. This was the first method used to increase gene delivery into keratinocytes and especially into Langerhans cells, which are specific dermal dendritic cells [5,33]. This induced a shift of the immune response towards the T_H2 pathway, inducing a humoral type of immune response.

The only approved adjuvants for human use are i) alum, aluminum salts and calcium salts, ii) MF 59, a microemulsion, composed of the mixture of surface active components and iii) virosomes, phospholipid particles carrying hemaglutinin and neuraminidase moieties on their surface. Many other adjuvant systems have proven their efficacy in human trials, however these were predominantly associated with non-tolerable toxicities.

Alum is the most commonly used adjuvant in humans. It is composed of aluminum hydroxide (AlO (OH)) or aluminum phosphate (Al - PO_4 in different ratios). This adjuvant system consists either of pre-formed particles or a gel (Alhydrogel). The antigen is adsorbed by electrostatic forces onto the surface of the Alum particles or particles form in-situ when the antigen is added to the gel [34,35]. Alum is used for a great variety of vaccines, such as the combined diphtheria-tetanus (Td) or the combination of five or six antigens in a preparation (Hexavac®, Aventis Pasteur MSD). The reproducible production of alum and its adsorption characteristics are a crucial part of the vaccine composition [36,37]. The mode of action is primarily the sustained desorption of the antigen or the toxoid from the Alum particles, resulting in an enduring

contact of the antigen with the immune system. Secondly, Alum enhances the immune response by activating the humoral immune response. Additionally, an increase in CTL reactions has been observed, compared to antigen preparation in solution [38]; however, strong IgE reactions and the toxicological concerns raised by use of aluminum represent the major drawbacks of these formulations [39,40].

Another mineral salt used for some vaccines is calcium phosphate, which has been shown to be better tolerated than alum, leading to fewer hypersensitivity reactions and an improved entrapment of antigens [41]. Other mineral, have also been investigated with minimal success, such as colloidal iron hydroxide, calcium chloride.

MF 59 (micro-fluidized emulsion) has been recently approved in Europe for use in subunit flu vaccines [42]. It is composed of 0.5 % Tween 80, 0.5 % Span and 4.3 % squalens forming an O/W-emulsion with a droplet size of 150 nm. This adjuvant is used in the influenza vaccines Fluad® (Chiron-Behring) und Addigripp® (Aventis Pasteur MSD). These vaccines are especially recommended for elderly, which frequently exhibited insufficient immune response using other conventional vaccines.

Virosomes are reconstituted influenza virus envelopes with inserted purified influenza glycoproteins (hemaglutinin and neuramindase). They are further representatives for particulate adjuvants. They serve as delivery tool for inactivated viruses [43]. These adjuvants are used in Berna Pharm´s hepatitis A vaccines (Epaxal®, Niddapharm / Havpur®, Chiron-Behring) [44]. Antigen presenting cells recognize the influenza epitopes and phagocytose the inactivated virus associated with the hepatitis antigen.

Other adjuvants retain only a scientific character. They are not used in humans because of frequent adverse reactions; some of these substances have been

approved for the use in animals. The effect of adjuvant mechanism and examples thereof are presented in Table 4.

Action	Adjuvant Type	Example	Benefit
Presentation	Amphiphilic molecules, complexes which interact with the immunogen	ISCOM's, liposomes, Quil, $Al(OH)_3$	Increased antibody response and duration
Targeting	Particulate adjuvants which bind the immunogen Carbohydrate adjuvants which target lectin receptors on macrophages and DC		Efficient use of the antigen: Antigen localization in the lymph nodes T_H1
Depot effect	W/O emulsions → short term Particles → long term	Microparticles Nanoparticles Oils, $Al(OH)_3$, gels	Prolonged antigen presentation Increased efficiency single dose vaccine ?
Danger signals	Oil emulsions, surface active agents, $Al(OH)_3$, IFN's, hsp		Tissue destruction, stress TCL binding on APC
Immuno - modulation	Small molecules or proteins which modify the cytokine network: co-stimulatory molecules, cytokines, chemokines	Complement CpGs, LPS cytokines	Up-regulation of the immune response. Selection of T_H1 or T_H2 balance Danger signal to innate immune cells. Inflammatory stimuli

Table 4: *Effects of different adjuvants on the immune system.*

Freund´s adjuvants are well-known and very potent immunostimulators. They can be divided into two groups, e.g. the complete (FCA) and the incomplete (FIA) adjuvants. Both consist of a mixture of a mineral oil with a surfactant

(Arlacel A). The complete adjuvant additionally contains mycobacteria components. The mechanism of action can be ascribed to a depot effect arising from the application of the antigen in a w/o emulsion. Further on, the activation of antigen presenting cells by the surfactant is achieved. The mycobacteria components in the FCA were found to be efficient adjuvant substances, due to the earlier mentioned CpG motives present in their genetic material. However, this complete Freund´s adjuvant was associated with severe inflammatory, painful and even harmful reactions [45].
Iscoms™ (Immune stimulating complexes) consist of saponins, phospholipides and cholesterol that form particles of approximately 40 nm into which the antigen can be incorporated. They induce T_H2 as well as T_H1 immune responses and are used for veterinary vaccines [46].

The adjuvants of interest in this work are particulate adjuvants, more specifically microparticles and nanoparticles prepared from biodegradable polymers.

The great advantage arising from these systems is their structural variability, the low toxicity arising from most of the synthetic, as well as natural polymers used and the possibility of further modification of the delivery system to target specific cells and tissues.

MICROPARTICLES

Microparticles are characterized primarily by their size, ranging from 1 to 1000 µm, although ideally > 100µm. In most cases, they are prepared of polymers. The mechanism of action of microparticles in vaccination is not entirely understood yet. However, similar to other particulate adjuvants they induce the activation of antigen presenting cells, due to the irritation of foreign particulate

matter in a size range similar to that of pathogens. It is further hypothesized that an inflammatory reaction results in danger signals. These signals attract antigen-presenting cells, which, in consequence, phagocytose the particles. Microparticles exhibiting diameters of less than 10 µm are susceptible to phagocytosis [47]. Additionally, the uptake of particles containing high concentrations of antigen results in higher levels of antigen delivered to phagocytes, including dendritic cells, as compared to the pinocytotic uptake of antigens in solution. After phagocytosis, antigen presenting cells, in particular dendritic cells, mature and migrate to the local lymph nodes [48]. Here, direct contact can be made with the residing lymphocytes [49]. Antigens associated to microparticles have been shown to induce cytotoxic T lymphocyte reactions in small animal models, in contrast to aluminum hydroxide adsorbed antigens [50].

The ideal microparticulate system should possess several characteristics: It should

- provide a depot effect of the antigen and its release over a certain time period, thus prolonging the presence of antigen in the organism.
- stabilize the antigen in the physiological environment against enzyme degradation.
- be easily and reproducibly formulated.
- be stable during storage.
- be free from toxic degradation products.
- be cost effective.

Preparation Techniques

The development of microparticle formulations using biodegradable polymers was described by Bungenberg de Jong in 1930 and numerous other groups [51-54]. The polymers used consisted of natural polymers, such as gelatin [55] and polysaccharides [56]. The disadvantages of natural products were their variability of the polymer quality, instabilities as well as safety concerns. Therefore, synthetic, biodegradable polymers have been preferentially studied for pharmaceutical use. The aim was to develop new drug delivery systems with defined and prolonged release profiles, especially for drugs susceptible to degradation in a physiological environment. The polymers for these formulations were strongly involved in the pharmacokinetics of the preparation. The type of polymer influences the rate of degradation, the type of degradation and the resulting degradation products and thereby the dug release. The most frequently used synthetic polymers were polyesters, poly(amides), poly(alyl-α-cyano acrylates) and poly(orthoesters). Polyesters have found a widespread use, due to their excellent biocompatibility and biodegradability leading to their approval by regulatory authorities [57,58]. Depending on the process parameters, the payload, the physicochemical drug characteristics, polymers and solvents, a multitude of structures could be developed.

The techniques most commonly used for the preparation of microparticles are spray drying, double emulsion methods and phase separation.

Spray Drying

The most popular method for microencapsulation is the spray drying technique. Particles of polyesters can be formulated by dissolving the polymer in a volatile organic solvent, such as methylene chloride or acetone. The polymer solution is nebulized inducing a fast evaporation of the solvent. The nozzle used for this purpose, the concentration and the viscosity of the solution, as well as the

boiling point of the solvent mainly determine the resulting size of the dried particles. Drugs can be incorporated by i) dissolving the drug in the organic solvent, ii) dispersing the solid, micronized drug in the polymer solution or iii) dispersing an aqueous solution of the drug in the polymer solution, either by an emulsion process or by high speed homogenization. As a result the drug becomes entrapped in the polymer matrix or it is covered by the polymer shell.

Important advantages of the spray drying technique are the ease of formulation and particle isolation. This results in parameters especially important for the industrial preparation, such as cost effectiveness, reproducibility and a widespread knowledge of the technique. Moreover, the process can be operated under sterile conditions, which is extremely favorable for the formulation of parenteral delivery devices. The temperatures used for the process depend on the boiling point of the solvent. For example, the preparation of PLGA particles from a methylene chloride solution can be achieved at a maximal temperature of 46°C. The polymer and the drug reach this temperature only for a very short period, as the evaporating solvent quickly cools the polymer solution and the droplets formed thereof. The exposure to organic solvents represents a noxious stress for many drugs, especially proteins. To stabilize the active components they can be lyophilized with cryoprotectants and dispersed a solid state in the polymer solution. DNA is less susceptible to degradation in organic solvents, therefore it is possible to use a dispersing process of the aqueous DNA solution in the organic solvent. A disadvantage of the spray drying technique is the relatively low yield, when small amounts of material are used. In large-scale productions this effect is reversed leading to very high yields.

Solvent Evaporation / Double Emulsion Methods (W/O/W)

These methods are based on the formation of small polymer solution droplets using a water immiscible organic solvent in an aqueous solution [59]. The double emulsion method has also been referred to as “in water drying method”.

The organic solvents mainly used for this process are methylene chloride and chloroform. When the solvent evaporation technique is used for the encapsulation of drugs, the drug substances have to be dissolved in the organic phase. For the encapsulation of drugs in aqueous medium a double emulsion method, e.g. a water - in oil - in water (W/O/W) technique has to be used. The primary emulsion is prepared by homogenizing a small volume of the aqueous drug solution into the organic solution containing the polymer by high-speed homogenization, sonication or vortexing. The primary dispersion is further rapidly injected into an aqueous stabilizer solution during simultaneous homogenization. Poly (vinyl alcohol) in concentrations ranging from 0.1 % to 0.5% is a frequently used stabilizer of the external phase. Others, such as poloxamers and gelatin have previously been used as well. The organic solvents have to exhibit a low solubility in water to permit the diffusion into the large external phase and further their evaporation.

During this process the polymer solidifies, resulting in microparticles containing small droplets of the aqueous drug solution. This implies that the solidification of the polymer occurs fast enough to inhibit the coalescence of the two, inner and external, aqueous phases. To achieve this, the volume of the external phase has to be large enough to rapidly extract the organic solvent from the polymer. The microparticles can be isolated and lyophilized for appropriate storage and stability. This technique provides the possibility to encapsulate hydrophilic drugs, including peptides and proteins, e.g. growth factors, LH-RH agonists [60-63], vaccines [64,65], as well as small molecular compounds, such as pseudoephedrine [66,67]. However, the interaction of proteins with hydrophobic surfaces may lead to alterations of their quaternary structure. The release kinetics of small hydrophilic molecules as well was difficult to control. Thus, this technique remains a challenge as it highly depends on the drug used.

Phase-Separation

The phase separation technique takes advantage of the decreasing solubility of a polymer in a solvent through the addition of a third non-solvent of this polymer. At a defined point of the process the polymer precipitates. This occurs particularly at surfaces and interphases. In this manner, the dispersed or dissolved drug is coated with the polymer. This method can be used for either hydrophilic compounds in aqueous solution, which are homogenized in the polymer solution, as well as for drugs that are dissolved or dispersed in a solid state in the polymer solution. The removal of the organic solvent, as well as the preservation of protein quaternary structures, however, have been shown to be a major difficulty related to that technique [68]. The preparation of organic, solvent-free systems using polymers such as chitosan for the preparation of microparticles may provide new possibilities [69].

Recently it has been demonstrated that microparticles have a great potential as DNA vaccine adjuvants [70-72]. Different strategies were pursued to exploit this property. Plasmid DNA was either encapsulated into the microparticles or was adsorbed onto the surface of cationic microparticles. Each system has shown both advantages and drawbacks.

DNA Encapsulation into Microparticles

Modern vaccines consist of proteins, peptides or polysaccharides, which have to be administered parenterally to circumvent degradation in the gastrointestinal tract. However, multiple injections have to be given to fully induce an effective immunization. Hence, the aim of many researchers was to develop a vaccine delivery system that would provide a modulation of antigen release, resulting in the `single shot´ vaccine [73,74]. These devices, in consequence, would exhibit prolonged immune responses [75-77]. Additionally, encapsulated antigens could be applied orally, thereby increasing the compliance and, more importantly, the

mucosal immunity. Particles < 10µm can be taken up by the gastrointestinal associated lymphatic tissue (GALT), which directly delivers the antigen to a mucosal tissue rich in dendritic cells [78]. Generally, the mucosa represents the main entry gate of pathogens into the organism. Thus, a strong mucosal immune response can directly neutralize the pathogen at the site of its entrance into the organism [70,79,80].

In most of the studies, DNA has been encapsulated into microparticles using a double emulsion technique, due to the hydrophilicity of the molecules. However, to obtain particle sizes suitable for GALT, as well as APC uptake high-speed homogenization or sonication had to be used. Compared to peptides and proteins, DNA is a relatively stable molecule in organic solvents, however it is degraded and looses its bioactivity rapidly when sheared [81,82]. Moreover, DNA encapsulated in poly (D,L-lactide-co-glycolide) (PLGA) polymers is exposed to an acidic environment created by glycolic- and lactic acid PLGA degradation products in the core of the particle [83,84]. Under these conditions, DNA is damaged by acid-catalyzed depurination and chain breaks [81]. Several methods have been proposed to circumvent the detrimental effects of encapsulation, such as i) the complexation of DNA with cationic polymers prior to encapsulation [85-87], ii) the homogenization in a frozen state (cryopreparation) [82], iii) the addition of buffering excipients [88] and iv) the preparation by self-emulsification processes [89]. Walter et al. moreover used a spray drying approach to prepare DNA microparticles adding buffering agents [86,88]. While the formulation concerns could possibly be solved, the effect of DNA release kinetics on the immune response has not been fully elucidated yet. The synchronization of the danger signal, practically the injection of microparticles, and DNA release were shown to be crucial on the induction of a potent immune response [90].

DNA Adsorption on the Surface of Cationic Microparticles

DNA can be adsorbed onto cationic surfaces, due to the overall negative charge arising from anionic phosphate groups, situated every 0.17 nm throughout the molecule [91,92]. Therefore, DNA can be associated with pre-formed cationic microparticles via electrostatic forces.

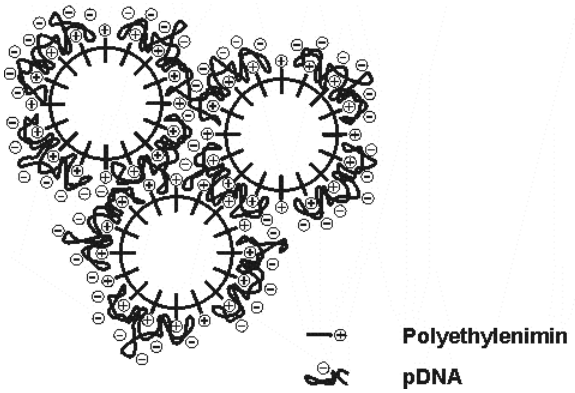

Fig.3: *Scheme of DNA adsorption on microparticles containing PEI (polyethylenimine) as a model cationic agent.*

In such a system DNA is only added to the preparation after the particle formation. Thus, DNA is not damaged during the formulation by high-speed homogenization or sonication.

Singh et al. successfully used these microparticles for in vivo immunization. The cationic surface properties of the microparticles were introduced by CTAB (Hexadecyltrimethylammonium-bromide), a cationic detergent, used as an external stabilizer [72]. CTAB thereby integrated into the surface of the particle. Immunizations against HIV gag and env proteins in small animals and rhesus macaques exhibited very promising results [93,94]. This system has different advantages over DNA encapsulation, i) DNA is not degraded during the formulation process, ii) a supplementary adjuvant effect arises from the CpG motives, presented on the surface of the system and iii) the interval between the injection of the particulate matter and release, or accessibility of DNA is much shorter than for encapsulated delivery systems.

NANOPARTICLES

Nanoparticles are characterized by definition, ranging from 1 to 1000 nm, however typically most formulations range from 1 to 500 nm in size. It has been shown by several groups that the cellular uptake, especially into non-phagocytic cells, is facilitated by small particle sizes [95]. Therefore, the preparation of nanoparticulate antigen delivery devices was hypothesized to achieve better immune reactions. The uptake in both phagocytic, as well as non-phagocytic cells, could increase the overall efficiency, as there are two modes of action proposed for the induction of DNA immunization. The two pathways are either the direct transfection of antigen presenting cells or 'cross-priming' by the transfection of cells. Similar to the microparticles, antigens can be either encapsulated into nanoparticles or adsorbed on the surface of cationic nanoparticles.

Preparation Techniques

Nanoparticle formation can be achieved using the same methods used to prepare microparticles by adjusting process parameters to obtain smaller particles.

Solvent Evaporation

It has been described previously that this technique requires on the formation of a disperse system composed of an immiscible organic polymer solution within an external stabilizer solution. The formulation of nanoparticles, as compared to microparticles, requires higher homogenization speeds or sonication, which both produce smaller droplets. Other parameters, such as the polymer concentration, exhibiting lower viscosities or surface active stabilizers in the external phase, facilitate the formation of nanoparticles. Hydrophilic drugs can be encapsulated

in nanoparticles using this method [96]; however, the resulting encapsulation efficiency of the hydrophilic substances is low, due to an increased diffusion of the hydrophilic molecules into the outer stabilizer phase. This diffusion increases, due to a delayed precipitation of diluted polymer solutions, a larger surface area of the nanoparticles and decreased diffusion barriers. Moreover, the high-energy sources used for the homogenization are detrimental to most protein, peptide and DNA drugs.

A modification of the solvent evaporation technique developed, is represented by the spontaneous emulsification / solvent diffusion method. In this case, a water soluble organic solvent is added to the water immiscible solvent containing the polymer [97]. Upon dispersion into the aqueous stabilizer solution, the water soluble organic compound diffuses into the aqueous phase. This leads to interphase turbulences resulting in smaller droplets and finally to the precipitation of the polymer. This method is effective for the encapsulation of lipophilic drugs. In contrast, hydrophilic drugs display low encapsulation efficiencies, due to their diffusion into the external phase. Several variations of this method exist, including the preparation in oil [98].

Solvent Displacement

This technique has further evolved from the spontaneous emulsification / solvent diffusion method [98]. The polymer is dissolved in a water soluble organic solvent, for example acetone, and the solution is injected into a stirred, aqueous, stabilizer solution. Upon contact of both solutions, the acetone immediately diffuses into the water, creating interphase turbulences. These interphase turbulences lead to the rupture of the interphase and to the formation of droplets that can further disrupt, resulting in smaller droplets containing the polymer. This process continues until precipitation of the polymer occurs. The described interfaced turbulences and disruption are known as Marangoni effect [99-101]. The turbulences in the interphase occur from convection of acetone as mass

transport into the aqueous phase and back into the vicinity of the interphase. The rate of acetone diffusion, and thus droplet disruption tendencies are dependent on the gradient of the diffusing solvent. As this system is in direct contact with water, the encapsulation of hydrophilic drugs generally results in very poor encapsulation efficiencies [101]. An advantage however is the quasi absence of high-speed homogenization and the absence of chlorated organic solvents.

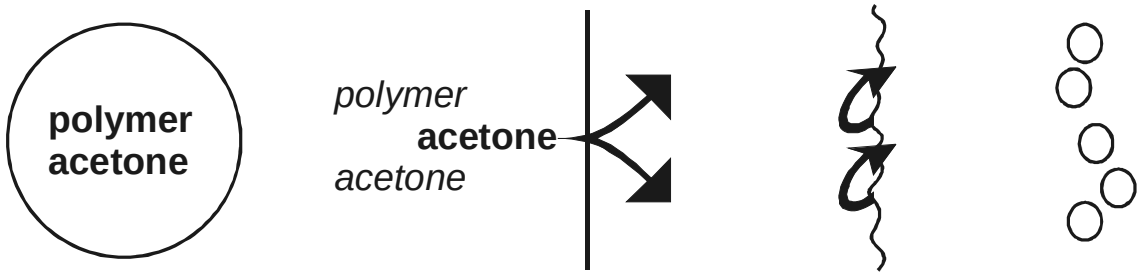

Fig.4: *Schematic process of nanoparticle formation by solvent displacement through the mass transport of the solvent acetone in the water phase.*

Salting out

This technique is based on the competition of compounds for solvents. A highly concentrated salt solution, containing a stabilizer, is added to a stirred acetone solution containing the polymer. The high salt concentration leads to a phase separation. Further addition of the salt solution leads to the reversal of the emulsion. The obtained oil-in-water emulsion is added to a larger volume of water, which finally results in the precipitation and complete diffusion of the organic solvent into the water [102]. Again, this process can only be efficiently used for the encapsulation of lipophilic substances.

DNA Encapsulation

The encapsulation of hydrophilic molecules into small hydrophobic polymer nanoparticles has been shown to be a rather inefficient process. As a result, several groups have encapsulated DNA into hydrophilic molecules such as chitosan. This process can be managed using a complexation-coacervation technique, where both a chitosan solution at pH 5 and a DNA solution are heated to 55°C and mixed together resulting in coacervation [103]. This technique circumvents the use of organic solvents, however replaces them with other potentially degrading conditions.

Several research groups have performed immunizations with these systems via oral administration. The immunizations resulted in elevated IgG antibodies against toxoplasma gonidii. However, a mucosal type of immunity indicated by IgA antibodies would be more beneficial [104]. Another group successfully modulated a peanut antigen-induced anaphylactic reaction in mice by converting high IgE levels to IgA and serum IgG antibodies using the oral allergen-gene immunization [105].

DNA Adsorption onto Nanoparticles

As discussed above, the encapsulation of hydrophilic molecules, such as DNA is difficult to achieve when using common nanoparticle preparation methods. Therefore, several research groups have adsorbed DNA onto the cationic surface of nanoparticles. The ideal ratio of DNA to nanoparticles depends on the nanoparticle size and charge. A prerequisite for the association of DNA with the particle surface through electrostatic interactions is the introduction of a cationic charge onto the nanoparticle surface. This has been achieved using CTAB, as was already mentioned for the preparation of cationic microparticles [72,106]. CTAB was internalized into the particles to generate a cationic surface of wax nanoparticles as well [107]. Other cationic polymers have recently been used for

the preparation of nanoparticles for DNA adsorption, e.g. poly ($_L$-lysine) graft-polysaccharides [108] and chitosan nanoparticles [109].

DNA can further be adsorbed onto inorganic, surface-modified nanoparticles. The preparation of surface - tethered DNA - gold-dendron nanoparticles [110], or amino modified silica nanoparticles [111] has been described. Aggregation and flocculation, resulting in impeded endocytosis is a frequently observed drawback of colloidal nanoparticle systems onto which DNA has been adsorbed [110,112]. Still, the immunizations with DNA nanoparticles of 300 nm have shown promising IgG levels, similar to those achieved with the CTAB modified microparticles studied by Singh et al. [72]. Cationic wax nanoparticles of 100 nm containing the endosomolytic agent, DOPE (dioleoyl phosphatidyl-ethanolamine), have demonstrated better immunization results as compared to naked DNA [113].

CONCLUSION

Numerous methods have been proposed to increase and modulate immune responses of DNA vaccines. Particulate, as well as non-particulate adjuvants have been investigated. Protective vaccination in small animal models has been successful, however, neither the traditional adjuvants nor new developments have successfully led to protection in human trials [18]. Therefore, further developments in vaccine adjuvants and certainly the well-advised combination of adjuvants, such as particulate adjuvants with non-particulate, immunomodulators is necessary to succeed. Recently, it has been proposed that the combination of DNA vaccines and protein antigen boosts would result in more promising immune responses [94]. However, DNA vaccine adjuvants leading to better gene delivery, depot effects, targeting of antigen presenting cells and activation of the desired type of immune response have to be further improved. The exact mechanism of such systems is not yet fully understood and

further investigations will be necessary to continue the progress and developments for more effective vaccines

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CHAPTER 2

AIMS AND SCOPE

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The great potential of DNA vaccines could not yet be used as efficiently, as hoped for in the beginning of DNA vaccine development in the 90's. Adjuvant systems are needed to (1) increase the DNA delivery, (2) achieve targeting to antigen presenting cells and (3) induce the activation of the immune response to reach protective levels. Although encapsulation of DNA in particulate systems has demonstrated promising results, numerous drawbacks of such systems persist.

Firstly, DNA is degraded by high-speed shear forces during the encapsulation into polymeric particles.

Further, DNA is exposed to acidic degradation products, such as lactic and glycolic acids of the polymer, which, in consequence, reduce the DNA bioactivity.

Finally, the gene delivery efficiency of most of the particulate systems is low.

We hypothesized that protecting DNA during the encapsulation process would increase DNA stability and bioactivity. The stabilizing agents should further modulate the DNA release kinetics from the formulation, to reduce DNA degradation by acidic polyester degradation products.

Adsorption onto pre-formed particles could circumvent DNA degradation during particle formation. As polyethylenimine (PEI) is a very efficient non-viral transfection agent we expected that the incorporation of PEI into particles could result in highly efficient DNA adsorption onto microparticles and gene delivery.

We hypothesized that the efficiency of DNA delivery systems could be greatly increased using polymers with specifically designed properties for that use.

Hence, to inhibit the acidification of the particle core the polymer should be fast degrading, it should ideally protect the DNA during and after the formulation of particles and promote gene delivery.

It was aimed to develop and characterize process parameters for DNA encapsulation using such a system. The formulation should then be optimized with respect to physico-chemical properties, such as small particle sizes and DNA stabilization, as well as efficient endocytosis and high gene delivery efficiencies by in vitro investigations.

Promising formulations should prove their potency as adjuvants for DNA immunization in vivo.

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CHAPTER 3

ENCAPSULATION OF DNA INTO MICROPARTICLES USING MODIFIED DOUBLE EMULSION METHODS AND SPRAY DRYING TECHNIQUES

SUMMARY

Recently, several research groups have shown the potential of microencapsulated DNA as adjuvants for DNA immunization [1]. The techniques generally used for the encapsulation of hydrophilic molecules into hydrophobic polymers are the modified double emulsion method and spray drying of water in oil dispersions. We investigated the possibility to encapsulate DNA avoiding shear forces which readily degrade DNA during these processes. DNA microparticles were prepared with polyethylenimine (PEI) as a complexing agent for DNA. Polycations are capable of stabilizing DNA against enzymatic, as well as mechanical degradation. Further, complexation was hypothesized to facilitate the encapsulation by reducing the size of the macromolecule. In this study, we additionally evaluated the possibility of encapsulating lyophilized DNA and lyophilized DNA / PEI complexes. For this purpose, we used the spray drying and double emulsion techniques. The size of the microparticles was characterized by laser diffractometry and the particles were visualized by scanning electron microscopy (SEM). DNA encapsulation efficiencies were investigated photometrically after complete hydrolysis of the particles. Finally, the DNA release characteristics from the particles were studied.

Particles with a size of < 10 µm which represents the threshold for phagocytic uptake, could be prepared with these techniques [2]. The encapsulation efficiency ranged from 100 % to 35 % for low theoretical DNA loadings. DNA complexation with PEI 25 kDa prior to the encapsulation process reduced the initial burst release of DNA for all techniques used. Spray-dried particles without PEI exhibited high burst releases, whereas double emulsion techniques showed continuous release rates.

INTRODUCTION

Microencapsulation of hydrophilic bio-molecules has gained increasing interest in the past decades as more peptide, protein, oligonucleotide and DNA drugs have become available for pharmaceutical use. These bio-molecules are frequently characterized by instabilities in physiological environments resulting in very short half-lives, especially due to their susceptibility to acidic or enzymatic degradation. The encapsulation in biodegradable polymers was found to be a promising approach to protect these drugs from nocuous factors. Further, the possibility of a controllable and sustained release, resulting in prolonged application intervals presents a major advantage. As most of the molecules of interest are hydrophilic, the favored method of encapsulation is the modified double emulsion method, referred to as “in water drying” [3]. This method allows the encapsulation of aqueous drug solutions within a hydrophobic polymer. One significant disadvantage of this process is the possible degradation of bio-molecules during the homogenization step of particle formation. Several groups have investigated alternative methods for homogenization, such as cryopreparation, used by Ando et al [4]. In addition to the harsh environment created during microparticle preparation, subsequent polymer degradation can also induce a destructive environment. For example, a typical feature of polyester microparticles is the decrease of the pH in the particle core. This results in the deterioration of encapsulated compounds [5,6].

Hence, we investigated the feasibility of several microencapsulation techniques, which, in our opinion, could be candidates for the protective encapsulation of bio-molecules, such as DNA. The encapsulation techniques used in this study were i) water in oil in water and ii) solid in oil in water techniques (Fig.1), as well as iii) spray drying water in oil and iv) solid in oil preparations (Fig.2). The effect of DNA complexation with PEI on the encapsulation efficiency and the DNA release were investigated in this study.

MATERIALS AND METHODS

Microparticle Preparation

Modified Double Emulsion Method

Particle formation was performed by a modified double emulsion technique [7]. 250 µl of an aqueous herring testes DNA (HT DNA) solution 2 mg/ml were homogenized in 5 ml methylene chloride solution containing 500 mg of a commercial PLGA (50:50), (Resomer® 503, Mw 41,000 g/ mol, specifications supplied by the manufacturer, Boehringer Ingelheim, Ingelheim, Germany). A scheme of the microparticle preparation is shown in Figure 1. The dispersion was formed by primary homogenization at 13,500 rpm for 30 s using an IKA 10G homogenizer (IKA, Staufen, Germany). This product was immediately injected into 400 ml of a stirred 0.1% poly (vinyl alcohol) (PVA) (Mowiol® 3-83, M_w 14,000; Clariant, Frankfurt) stabilizer solution in ultrapure water at pH 7. This final dispersion was formed using an IKA 25F homogenizer (IKA, Staufen, Germany) at 20,500 rpm for 30 sec. The particle suspension was stirred with a propeller mixer at 200 rpm for three hours for methylene chloride extraction and evaporation. Microparticles were isolated by centrifugation at 10,000 rpm for 10 min in a Sorvall high-speed centrifuge (LB-5, Haereus, Hanau, Germany). DNA/PEI 25 kDa (BASF, Ludwigshafen, Germany) complexes were encapsulated by the same method. The complexes were prepared in ultrapure water at a nitrogen to phosphate (N/P) ratios of 5 and 10. The PEI/DNA complexes in 250 µl water were dispersed in the organic polymer solution for the formation of microparticles. The centrifuged particles were re-suspended and washed three times and finally redispersed in 2 - 5 ml of ultrapure water. The preparation was lyophilized in a Modulyo freeze dryer (Edwards, Sussex, UK). Microparticles were stored at 4 °C until further use.

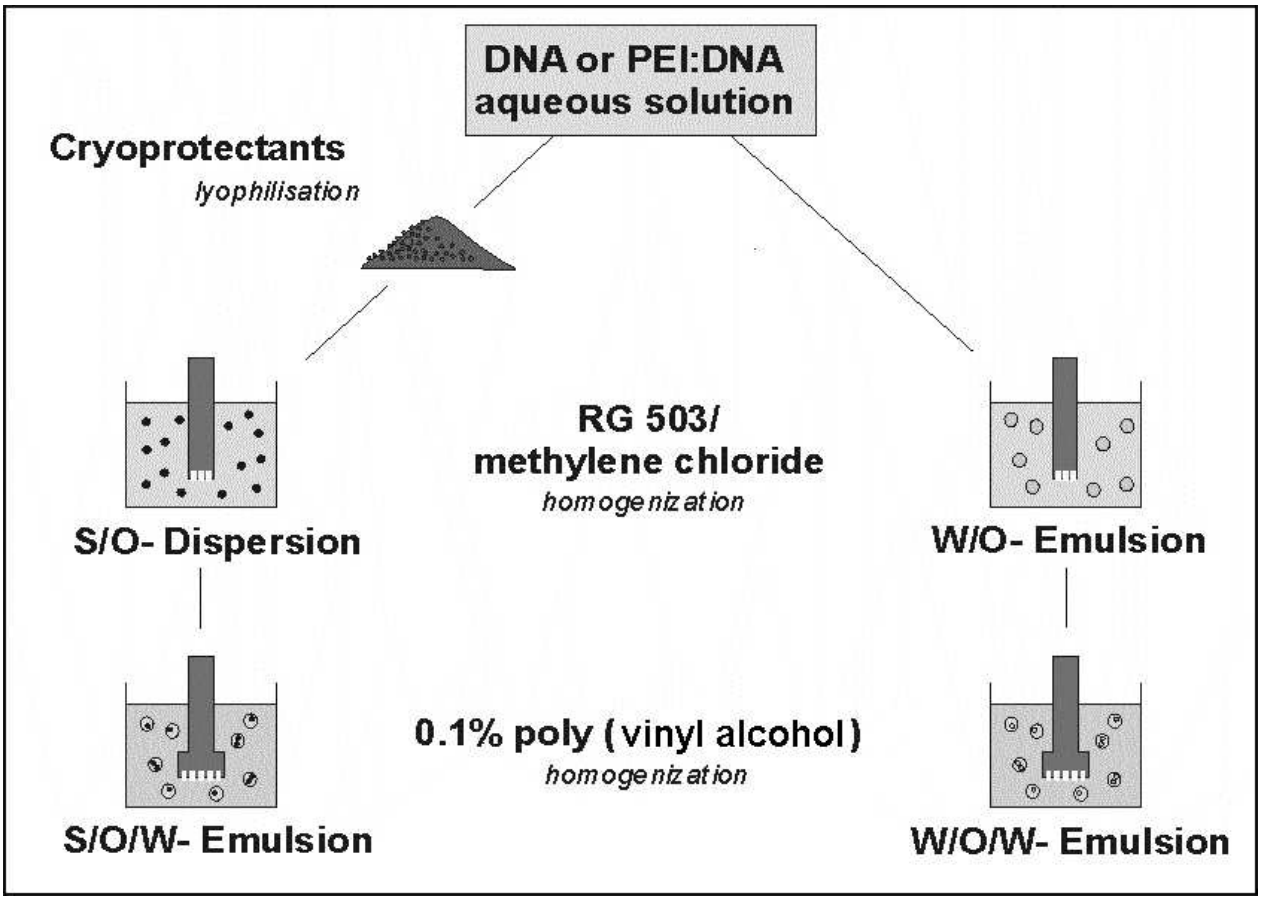

Fig.1: *Scheme of the modified double emulsion method (W/O/W) and the solid in oil in water method (S/O/W).*

Solid in Oil in Water

Solid in oil in water microparticles were prepared by homogenizing dispersions of lyophilized HT DNA or PEI/DNA complexes combined with the lyoprotectants, glycin (Merck, Darmstadt, Germany) or lactose (Hoechst, Frankfurt, Germany)(Fig.1).

For that purpose, a 0.1% DNA solution containing either 5 % lactose or 5 % glycin was lyophilized. In parallel, PEI 25 kDa / DNA complexes at a N/P ratio of 5 were lyophilized with a 0.1% DNA content in 5% lactose or glycin aqueous solutions. Both formulations were micronized in a mortar for 20 minutes. The powders were dispersed in 2 ml of the organic methylene chloride solution containing 200 mg RG 503. The amount of the powdered substance was

calculated to obtain a theoretical loading of 0.1 % DNA / polymer [$^{m}/_{m}$]. The dispersion was prepared by homogenizing the solids in the methylene chloride solution using an IKA 10G homogenizer (IKA, Staufen, Germany) at 13,500 rpm for 30 seconds. The product was immediately injected into 200 ml of a stirred 0.1 % PVA stabilizer solution in ultrapure water at pH 7. The final dispersion was formed with an IKA 25F homogenizer (IKA, Staufen, Germany) at 20,500 rpm for 30 sec. The particle suspension was finally stirred at 200 rpm for three hours with a propeller mixer in order to extract and evaporate the methylene chloride. Microparticles were isolated by centrifugation at 10,000 rpm in a Sorvall high-speed centrifuge LB-5 for 10 minutes. The particles were re-suspended and washed three times and redispersed in 2 – 5 ml volume of ultrapure water. Lyophilization was performed in a Modulyo freeze dryer. The microparticles were stored at 4 °C until further use.

Spray Drying

Microparticles were formed by spray drying either a water in oil dispersion or a solid in oil dispersion using a Büchi 190 laboratory Mini Spray dryer (Büchi, Flawil, Switzerland) (Fig.2). For the water in oil method, 1.47 ml aqueous phase was used either containing 1 mg/ml of an aqueous DNA solution or 1 mg/ml DNA complexed with PEI at a N/P ratio of 5. For the solid in oil method, powdered DNA or PEI/DNA complexes containing either lactose or glycin were used. Both of these internal phases were dispersed in 39.62 ml of a methylene chloride solution of 1.47 g RG 503. The dispersions were formed by high-speed homogenization with an IKA 25F homogenizer (IKA, Staufen, Germany) at 13,500 rpm for 30 seconds. The resulting dispersion was stirred continuously and was spray dried immediately using a 0.5 mm outer mixing two-fluid nozzle and an inlet temperature of 45-46 °C. The outlet temperature was set to 32-35 °C by the pump rate, which was set to the lowest possible velocity.

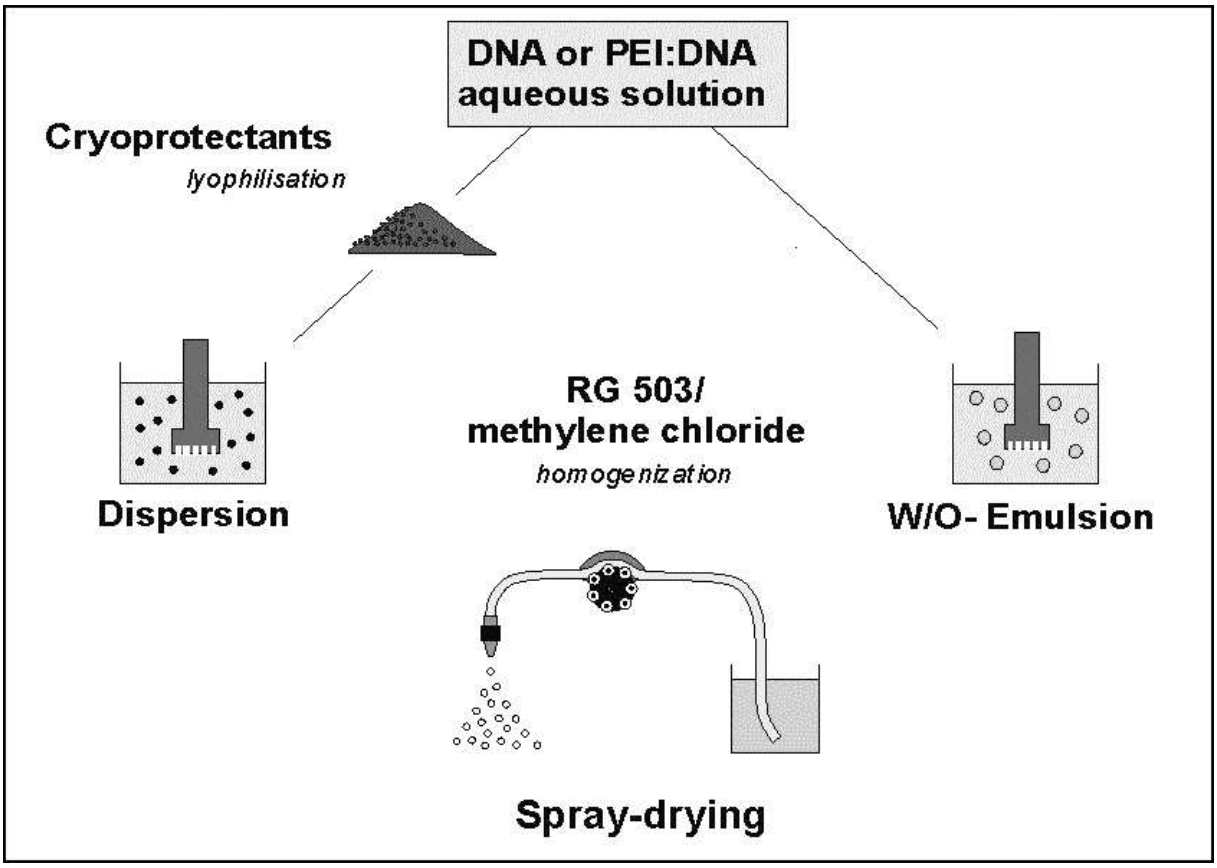

Fig.2: *Scheme of the spray drying techniques using a water in oil (W/O) and a solid in oil (S/O) technique for DNA encapsulation.*

The spray flow, representing the velocity of the air transport in the spray cylinder, was set to its maximum with the aspirator settings of 5 / 20, in order to reduce the escape of small particles through the cyclon. Particles were collected, lyophilized for remaining water elimination and stored at 4°C until use.

Particle Size

The microparticle size was analyzed by laser diffractometry in a Mastersizer X (Malvern Instruments, Germany) equipped with a magnetically stirred cell. Measurements were carried out with a 100 mm lens, covering a particle size range of 0.5 – 180 µm. The samples were diluted with 0.1% Tween 20 in ultrapure water. For data analysis the refractive index of ultrapure water (1.33)

was used. The calculation of particle sizes was carried out using the standard modus of the Malvern software according to the theory of Mie. The weighted average of the volume distribution [4.3] was used to describe the particle size. D [4.3] is defined by Σnd4 / Σnd3 (n = number of particles in each area of particle sizes, d = medium particle diameter in the area of particle sizes). All measurements were carried out in triplicate.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed using a Hitachi S 510 (Hitachi, Tokyo, Japan) in vacuum at a voltage of 25 kV after gold sputter coating using an Edwards/ Kniese Sputter Coater S150 (Edwards, Germany).

DNA Encapsulation Efficiency

DNA encapsulation efficiency was measured by complete hydrolysis of the weighted particles in 1 ml 0.4 N NaOH. The concentration of DNA was measured photometrically using a Shimadzu UV-160 (Shimadzu, Duisburg, Germany) at a wavelength of 260 nm. Concentrations were calculated from calibration curves of degraded DNA.

DNA Release

DNA release was studied by suspending triplicates of 5- 10 mg of microparticles exactly weighted in 1 ml of ultrapure water at pH 7. Water was chosen to circumvent solubility problems of DNA in buffers containing divalent ions. The samples were incubated at 37°C and agitated once a day. For DNA release analysis, the sample triplicates were centrifuged at 2000 rpm for 5 min in an Eppendorf 5415C centrifuge (Wesseling, Germany), according to Gebredikan et al. [8]. The supernatant was analyzed for DNA at 260 nm.

RESULTS AND DISCUSSION

Microparticle size for gene delivery to antigen presenting cells or to the mucosal associated lymphocyte tissue is limited to 10 µm, which represents the threshold for phagocytic uptake [2]. Therefore, microparticles for this purpose have to be smaller that those usually formulated for controlled drug delivery [9]. In most cases, this implies the use of high-speed shear forces to better disrupt the aqueous phase, as well as the oil droplets. As a consequence, the effectiveness of encapsulation is reduced resulting from an increased diffusion of the hydrophilic compound into the external phase. Other possibilities to reduce the particle size, such as the use of a less viscous polymer solution, higher concentrations of stabilizers in the external phase cannot prevent this phenomenon [10,11]. Therefore, we investigated several methods of microparticle preparation with the aim of formulating microparticles <10 µm exhibiting efficient drug loadings and sustained drug release profiles.

The size of the microparticles obtained by spray drying were similar, independently of the formulation variation in this study, ranging from 2.55 µm to 8,15 µm (Table 1). This is in accordance with the literature [12,13]. The particle size mainly depends on the viscosity of the organic phase, the boiling point of the organic solvent and the geometry of the nozzle [11].

W/O/W microparticles exhibited particle sizes smaller than 10 µm making them suitable for phagocytosis. In contrast, S/O/W microparticles exhibited diameters larger 10 µm. The microparticles prepared using solid in oil (S/O) techniques did not display increased diameters, although the micronized solid components were in part larger than the polyester particles (Fig.3). These larger solid fragments however did not appear to influence the size measurement. This could either be attributed to the dissolution of uncoated particles in the aqueous medium during laser diffractometry or, alternatively, this phenomenon could be attributed to the very low amount of the larger, polymer coated particles.

Microparticle		Method	Size [µm]	Content [%]
DNA	0.1 %	W/O	4.74 ± 0.15	0.104 ± 0.011
DNA/PEI	0.1 %	W/O	8.15 ± 1.09	0.111 ± 0.011
DNA	1 %	W/O	5.53 ± 0.42	0.665 ± 0.011
DNA/PEI (5)	1 %	W/O	2.87 ± 0.09	0.681 ± 0.016
DNA/PEI (10)	1 %	W/O	5.15 ± 1,54	0.673 ± 0.048
DNA / glycin	0.1%	S/O	3.32 ± 0.03	0.104 ± 0.011
DNA / lactose	0.1%	S/O	2.55 ± 0.18	0.049 ± 0.005
DNA/PEI / glycin	0.1%	S/O	3.97 ± 0.01	0.105 ± 0.014
DNA/PEI / lactose	0.1%	S/O	4.03 ± 0.17	0.035 ± 0.004
DNA/PEI / glycin	1%	S/O	6.68 ± 0.82	0.683 ± 0.027
DNA	0.1%	W/O/W	8.61 ± 2.87	0.086 ± 0.001
DNA/PEI (5)	0.1%	W/O/W	4.09 ± 1.01	0.112 ± 0.007
DNA/PEI (10)	0.1%	W/O/W	6.09 ± 0.66	0.085 ± 0.004
DNA / glycin	0.1%	S/O/W	15.84 ± 2.89	0.048 ± 0.008
DNA / lactose	0.1%	S/O/W	16.55 ± 1.88	0.107 ± 0.007
DNA/PEI / glycin	0.1%	S/O/W	7.44 ± 0.03	0.085 ± 0.003
DNA/PEI / lactose	0.1%	S/O/W	13.96 ± 8.73	0.064 ± 0.01

***Table 1**: Characterization of microparticles formulated using the four preparation techniques described. Particle size was measured by laser diffractometry in triplicate and described by the weighted average of the volume distribution [4.3] as average and standard deviation. HT DNA content was assessed by photometric measurement at 260 nm after particle hydrolysis in 0.4 N NaOH and presented as average and standard deviation.*

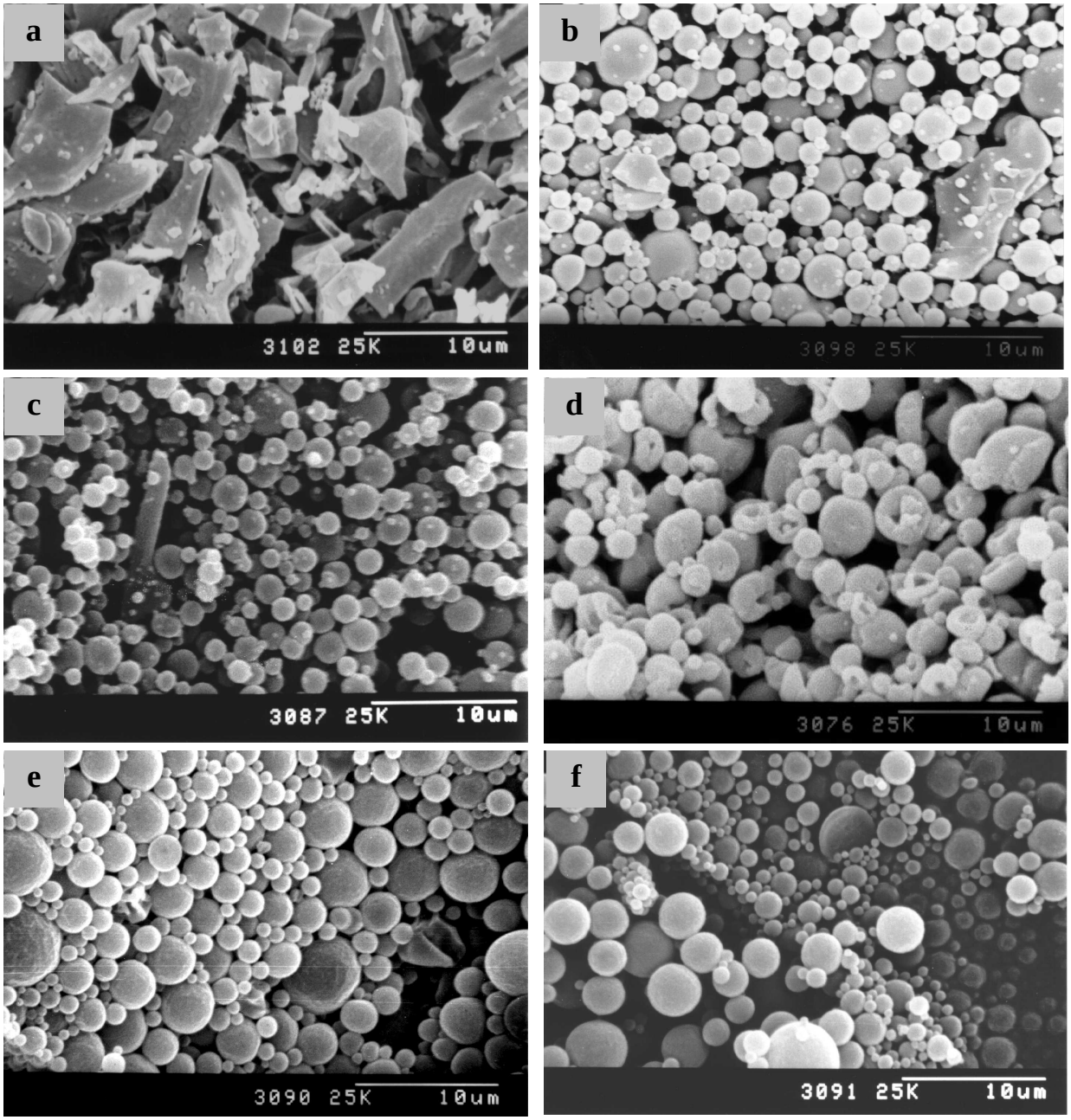

Fig.3: *SEM micrographs of 0.1% DNA loaded RG 503 (PLGA) particles. (a) PEI/DNA complexes lyophilized in a 5 % glycin solution. (b) S/O spray dried microparticles with DNA lyophilized in glycin, (c) S/O spray dried microparticles of PEI/DNA complexes lyophilized in glycin, (d) W/O 1% DNA spray dried particles with PEI/DNA complexes in solution. (e) W/O/W particles of DNA and (f) W/O/W particles of PEI/ DNA complexes.*

The SEM micrographs showed large, non-spherical components mixed with the microparticles. This finding indicates an incomplete incorporation of the solid, due to insufficient size reduction. Other methods of particle size reduction, such as stirred ball mills would potentially be more appropriate. We considered that particles may have also been disrupted by the high-speed homogenization process. All other microparticle sizes measured by laser diffractometry were confirmed by SEM. Furthermore, SEM revealed microparticles with regular shapes and a smooth surfaces.

The DNA content of all preparations was assessed by complete hydrolysis of the particles (Table 1). A low theoretical drug loading of 0.1%, resulted, as expected, in efficient encapsulation of most of the formulations, using the water in oil in water (W/O/W) technique [14]. The S/O/W preparations demonstrated encapsulation efficiencies ranging from 64 % of theoretical drug loading to complete encapsulation. However, the S/O/W-glycin-DNA particles had low encapsulation efficiencies. This was attributed to the incomplete encapsulation of the lyophilized components. The microparticles formulated by spray drying exhibited high drug loading efficiencies with the exception of the lactose solids.

The DNA release was investigated for all preparations and presented as the fraction of DNA released into the medium. The release properties of W/O spray dried particles depended, to a great extent on the formulation parameters. For example, the microparticles prepared with 1% theoretical DNA loading released the complete dose within hours (Fig.4). In comparison, microparticles with a 0.1% DNA theoretical loading exhibited an initial burst of approximately 40% and a slow release, reaching a maximal level of 70 %. High burst effects of small spray dried microparticles prepared from similar polyesters as used in this study have been previously reported [15,16]. Interestingly, the complexation with PEI 25 kDa resulted in an 80% retention of the DNA within the particle formulation.

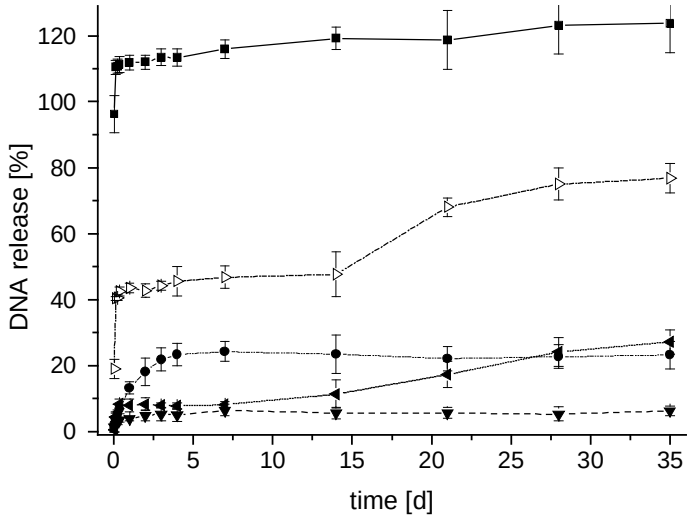

Fig.4: *HT DNA release from RG 503 microparticles using a water in oil spray drying technique.* ▷ *DNA 0.1%;* ◀ *PEI/DNA 0.1% (N/P= 5);* ■ *DNA 1%;* ▼ *PEI /DNA 1% (N/P= 5)* ● *PEI/DNA 1% (N/P = 10)*

This was surprising, as it was assumed that polymer degradation would be accelerated by PEI, due to a basic catalyzed polyester hydrolysis [17].

One possible explanation may be the enhanced dispersion of the DNA/PEI complex in the polymeric matrix, due to the lower hydrophilicity of the complex or due to its reduced size. Another reason for the low release could be the aggregation of the DNA complexes in the particle core, which could arise from the swelling of PEI following its protonation in the acidic core of the particle. Due to strong electrostatic interactions of the complex, DNA will not get separated from PEI and retained in the particles.

The S/O spray dried formulations with a theoretical DNA loading of 0.1% were compared to the 0.1% W/O particles (Fig.5). Both the lactose and glycin containing DNA microparticles released DNA instantly. This effect was explained by the high content of water soluble components, comparable to the 1% DNA W/O formulation.

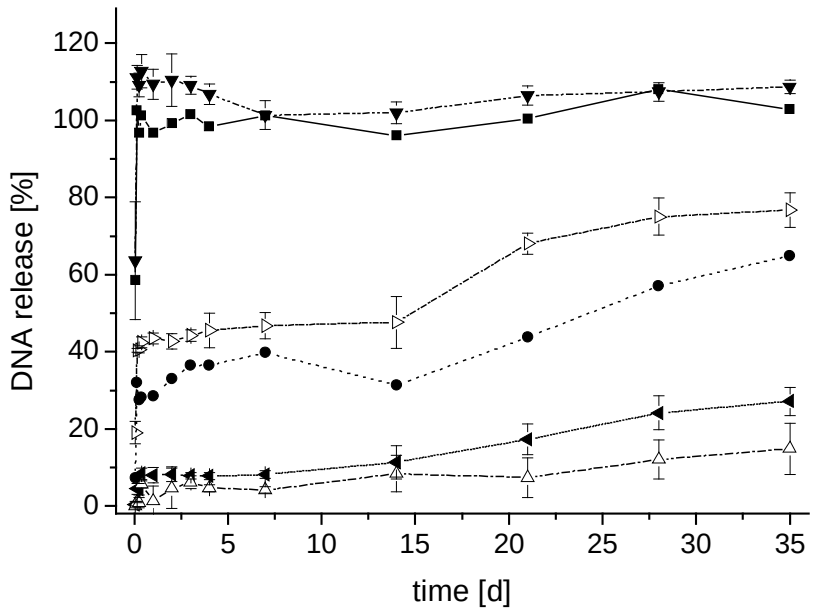

Fig.5: *HT DNA release from RG 503 microparticles prepared by a solid in oil spray drying technique with a theoretical DNA loading of 0.1%.* ■ *DNA lactose;* ● *PEI/DNA lactose;* ▼ *DNA glycin;* ∆ *PEI/DNA glycin;* ▷ *DNA (W/O);* ◀ *PEI/DNA (W/O)*

The high content of small, water soluble molecules resulted in the formation of large pores in the particle followed by an immediate release of the DNA [18]. The formulations with DNA/PEI complexes, in contrast, again exhibited slower DNA release profiles. The lactose DNA/PEI formulation showed a faster release as compared to the glycin containing particles. This could be explained by a lower interaction of the sugar, lactose, with the DNA/PEI complex, as compared to that with the amino acid.

The S/O/W and W/O/W microparticles were characterized by relatively constant release kinetics, exhibiting low burst releases of approximately 20% (Fig.6). The W/O/W formulations, either DNA alone or complexed with PEI, were characterized by very slow release profiles. This can be explained by the good

polymer shell formation over of the inner water droplets. Surprisingly, the glycin-S/O/W particles exhibited a faster release of the complexed DNA compared to DNA alone with the amino acid.

Although, these release kinetics appear to be the most suitable, the particle sizes of these formulations were larger than the phagocytosis threshold.

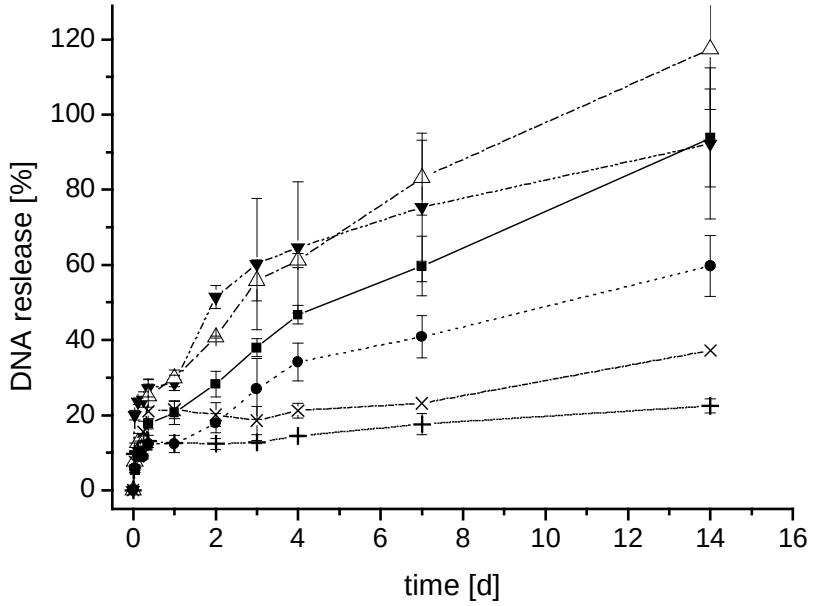

Fig.6: *HT DNA release of RG 503 microparticles using a solid in oil in water encapsulation method and the water in oil in water method for 0.1% DNA encapsulation.* ■ *DNA lactose;* ● *PEI/DNA lactose;* ▼ *DNA glycin;* ∆ *PEI/DNA glycin;* **+** *PEI/DNA W/O/W;* ***x*** *DNA W/O/W*

CONCLUSIONS

In this study we investigated potential techniques for DNA microencapsulation and possibilities to circumvent shear forces by lyophilizing the unstable component prior to its exposure. Still, the size of solid particles has to be further reduced before homogenous particles and efficient encapsulation could be

achieved. The concentration of the lyoprotectants should be reduced to decrease the amount of water soluble components in the spray dried formulation in order to allow a higher drug loading. In this study, PEI 25 kDa obviously acted as retardation agent, in contrast to results reported by de Rosa et al. [17], who observed increased oligonucleotide release levels after PEI complexation. The extremely high N/P ratios (N/P 15 and 45) used in this study could be one explanation for this discrepancy. This could increase the DNA release, possibly by inducing pore formation or catalysis of polyester degradation. Further the decomplexation rate of oligonucleotides compared to DNA is considerably higher. Although the S/O/W and the W/O/W formulations exhibited the most regular release properties, the S/O formulations showed the greatest potential to modulate the release kinetics of DNA by allowing the addition of complexing agents and lyoprotectants.

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CHAPTER 4

CATIONIC MICROPARTICLES CONSISTING OF POLY (LACTIDE-CO-GLYCOLIDE) AND POLYETHYLENIMINE AS PARENTERAL CARRIERS SYSTEMS FOR DNA VACCINATION

SUMMARY

Microparticles were formulated by blending the polymer, poly (lactide-co-glycolide) (PLGA) (50:50), with different amounts of cationic agents, either PEI 25 kDa (polyethylenimine) or CTAB (hexadecyltrimethylammonium-bromide). The aim was to create microparticles with cationic surface characteristics for DNA adsorption. Microparticles formulated with 10% PEI exhibited a highly positive ζ-potential, small particle sizes, in contrast to particles prepared with CTAB, which showed highly aggregated structures in the scanning electron micrographs. PEI 10% microparticles very efficiently adsorbed DNA and protected DNA from enzyme degradation.

Microparticles with up to 10% PEI did not exhibit LDH levels considered as toxic, whereas CTAB particles showed higher membrane toxicities. Gene delivery efficiencies were assessed via quantification of the reporter gene, luciferase, and compared to PEI/DNA complexes. The PEI formulations with 10% and 50% PEI exhibited elevated transfection efficiencies. The mechanism of gene delivery to non-phagocytic cells was studied via covalent fluorescence labeling of both the DNA and PEI by confocal microscopy. In vivo immunizations were performed with plasmids encoding *Listeria monocytogenes* antigens adsorbed onto PEI 10% microparticles. The efficiency was tested by the challenge with an i.v. injection of a lethal dose of the *Listeria monocytogenes*. Mice immunized with three booster injections of 10 µg DNA adsorbed onto the particle formulation exhibited a slightly better protection than naked DNA.

INTRODUCTION

Vaccines can be considered to be one of the most effective developments in modern medicine. A considerable drawback of non-live vaccines, however, is their lack of effectiveness against intracellular and viral pathogens, such as tuberculosis or HIV. A strong immune response against these pathogens depends on the induction of a potent cellular immune response and cytotoxic T-lymphocyte (CTL) reactions. During the past decade, DNA vaccination has been increasingly employed in an attempt to achieve simpler, safer, and more effective CTL reactions. DNA vaccination involves the inoculation with an expression vector that encodes an antigenic protein. The encoded antigen is then produced in situ and elicits an immune response [1]. Several studies have shown that the induction of more efficient immune responses from DNA vaccination could be generated by the use of adjuvant delivery systems [2]. More specifically, the adsorption of DNA on the surface of pre-formed cationic microparticles resulted in remarkable immune responses [3]. The cationic surface charge of these microparticles was obtained by the incorporation of a cationic detergent, CTAB, into the surface of the microparticles during their preparation. CTAB, was primarily used for DNA isolation from bacteria and plants by precipitation [4].

A microparticulate DNA delivery system based on the adsorption of DNA onto its surface has the clear advantage of i) circumventing the degrading effects on DNA during particle preparation ii) facilitating a rapid delivery of DNA to targeted antigen presenting cells and iii) providing an additional adjuvant effect by the presence of bacterial CpG units of the plasmid on the surface of the delivery system.

In the present study, we investigated the potential of PEI to form cationic microparticles by direct internalization of the polycation into the PLGA matrix. CTAB was also directly mixed with the PLGA (RG 502H) solution. PEI 25 kDa

is one of the most powerful non-viral transfection agents used in vitro and in vivo [5]. Thus, we hypothesized that the adsorption efficiency and the gene delivery would be increased with such a system. The microparticles were characterized with regard to their physicochemical properties, their stabilizing effects on DNA integrity, in vitro characterization of the membrane toxicity and gene delivery. Finally, the most effective in vitro delivery system was used for in vivo immunization against the intracellular bacterium, *Listeria monocytogenes*, to assess the induction of a protecting immune response.

MATERIALS AND METHODS

Materials and DNA

The commercially available poly(lactide-co-glycolide) (PLGA) (50:50), Resomer® 502H, (M_w 15,200, uncapped end-groups specifications supplied by the manufacturer) and PLGA (50:50), Resomer® 505 (M_w 80,000, specifications supplied by the manufacturer) were purchased from Boehringer Ingelheim (Ingelheim, Germany). Partially hydrolyzed poly(vinyl-alcohol) (PVA) (Mowiol® 3-83, M_w 14,000) was purchased from Clariant (Frankfurt, Germany). Polyethylenimine (PEI) 25 kDa, was purchased from BASF (Ludwigshafen, Germany) and stored under exclusion of humidity. Hexadecyltrimethyl-ammonium-bromide (CTAB) was purchased from Fluka (Buchs, Germany). Plasmid DNA, pLuc-CMV, a luciferase encoding plasmid, preceded by a nuclear location signal under the control of a CMV promoter, was kindly provided by Chiron (Emeryville, Ca) and amplified by PlasmidFactory, (Bielefeld, Germany). All pLuc-CMV probes used were from one endotoxin free batch in TE-Buffer pH 8 and stored at – 80°C until use. All other chemicals were of analytical grade.

Plasmid DNAs encoding p60 named pCiap, listeriolysin O (LLO) named pClisA, and non-hemolytic, mutant LLO named pChly492 were constructed by Fensterle et al. and effectively used for DNA vaccination by gene gun immunization [6,7]. Briefly, wild-type LLO gene and p60 gene of *Listeria monocytogenes* without the bacterial signal sequence were amplified by polymerase chain reaction (PCR) and inserted into EcoRI/XbaI site and XhoI/XbaI site of pCI mammalian expression vector (Promega, Madison, WI, U.S.A.), respectively. *L. monocytogenes* strain BUG337 encoding an LLO version with a single amino acid (a. a.) exchange at the a. a position 492 (Trp-492-Ala) was kindly provided by Dr. P. Cossart [8]. The mutant LLO gene was amplified from genomic DNA of *L. monocytogenes* strain BUG337 by PCR, and integrated into XhoI/XbaI site of pCI vector.

Particle Preparation

Microparticles were prepared by a modified double emulsion procedure under aseptic conditions. Briefly, the cationic agent (PEI/CTAB) was dissolved in methylene chloride and dispersed in a PLGA solution in methylene chloride resulting in a final volume of 10 ml. The amount of cationic agent added to the polymer was specified as % of the PLGA mass. PBS buffer of the internal phase was added to the CTAB / methylene chloride solution for complete dissolution. Aside from the incorporation of cationic agents into the organic polymer solution, microparticles were also prepared in aqueous solutions containing CTAB as stabilizer. Microparticle preparation was performed by initial homogenization of 1 ml PBS within the polymer solution at 13,500 rpm for 30 s, using an IKA 10G homogenizer (IKA, Staufen, Germany). The preparation was immediately injected into 50 ml of a stirred stabilizer solution (PVA 0.5% or CTAB 0.5%) and homogenized at 20,500 rpm for further 30 s, using the IKA 25F homogenizer. The particle suspension was stirred at 200 rpm for methylene chloride evaporation over 12 hours in a laminar air flow. Particles were isolated by

centrifugation at 4°C in a Sorvall high-speed centrifuge (LB-5, Haereus, Hanau, Germany) at 6,000 rpm for 20 min. The pellet was re-suspended and washed three times. A sterile 5% sucrose solution in distilled water was used, to wash the particles and for the final lyophilization in a Beta II lyophilizer (Christ, Osterode, Germany). Particles were stored at 4°C until use.

Particle Size

The particle sizes were analyzed by laser diffractometry using a Mastersizer X (Malvern Instruments, Herrenberg, Germany) in a stirred cell, with a volume of 15 ml. The measurements were carried out with a 100 mm lens, covering a particle size range of 0.5 – 180 µm. The samples were diluted in ultrapure water for measurement within the required range of obscuration. For data analysis the refractive index of ultrapure water (1.33) was used. The calculation of particle size was carried out using the standard modus of the Malvern software according to the theory of Mie. The weighted average of the volume distribution [4.3] was used to describe the particle size. D [4.3] is defined by $\Sigma nd4 / \Sigma nd3$ (n = number of particles in each area of particle sizes, d = medium particle diameter in the area of particle sizes). All measurements were carried out in triplicate.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed with a CamScan 4 (Cambridge, UK) after gold sputter coating using an AUTO 306 (Edwards, UK). High resolution transmission electron microscopy imaging (TEM) was performed after cryo-sectioning of the nanoparticles with a JEM 3010 (Jeol, Japan) on a collodium grid.

Zeta Potential Measurement

ζ-potential measurements were carried out using the Zetasizer 4 (Malvern Instruments, Germany) by electrophoretic light scattering after re-suspending the lyophilized particles in low ionic strength buffers (I=0.01) with varying pH from 3 to 8. Samples were diluted to a defined count rate interval of 400 – 800 kcps. Electrophoretic light scattering was performed in a AZ 104 cell. Average ζ-potential values were calculated from the data of 3 runs. The instrument was calibrated with a Malvern –50 mV transfer standard.

DNA Adsorption Efficiency

DNA was adsorbed onto the microparticles using a 0.5 mg/ml DNA solution to obtain a theoretical DNA loading of 1%. The particle suspension containing the DNA resulted in a final volume of 400µl. The particles were centrifuged at 10,000 rpm in an Eppendorf 5415C centrifuge (Wesseling, Germany) for 10 min after one hour of incubation. The adsorption efficiencies were calculated from the remaining DNA in the supernatant by UV measurement in a Shimadzu UV-160 (Shimadzu, Duisburg, Germany) at 260nm.

Lactate Dehydrogenase Release

The release of lactate dehydrogenase (LDH) was performed to characterize the membrane toxicity of the microparticle formulations. L929 mouse fibroblasts (DSMZ, Braunschweig, Germany) were seeded at a density of 50,000 cells per 2 ml in 12 well culture dishes (Nunc, Wiesbaden, Germany) and grown for 24 h prior to the incubation with the particles, according to the supplier's recommendations. The cells were washed twice with PBS buffer (0.1 M, pH 7.4). Subsequently, the cells were incubated with 2 ml of a microparticle suspension containing 1 mg particles /ml PBS buffer. Blank PBS buffer and a 0.1 % Triton-X 100 solution in PBS buffer were used as controls. 100 µl samples were withdrawn after 180 min

and processed according to the manufacturer's instructions (Sigma Diagnostics). All sample values were normalized relative to Triton-X values and expressed as relative LDH release in [%]. Each sample was performed in triplicate. The differences of all population means were analyzed by a two-sample t-test and one-way ANOVA at the 0.05 level.

DNase Stability

DNA stability was studied using 100 µl aliquots of the microparticle suspensions containing 1 µg pDNA. The probes were incubated with 12.25 µl DNase buffer 10x (1M Na-acetate, 50 mM $MgCl_2$) and 2.5 µl DNase I solution (DNase I Boehringer Mannheim, Germany) (50 I.U. / ml in 50 mM Tris-HCl pH 8, 100 mM KCl). The reaction was terminated by adding 5.7 µl EDTA solution (0.5M, pH8). The probes were freeze-dried and stored at –20°C until use. At the time of DNA analysis, the dried probes were incubated for one hour in 10 µl TBE-buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) containing 50 I.U heparin (Serva, Heidelberg, Germany). Further, 10 µl Roti-phenol® (Roth, Karlsruhe, Germany) were added and incubated for additional 2 hours at room temperature. Before the application onto a 1% agarose gel 5 µl glycerol were added to the emulsion. Untreated DNA was applied to the gel as a reference. Electrophoresis (Blue Marine 200, Serva, Germany) was carried out at 100 V for two hours in TBE-buffer. Ethidium bromide was included in all gels to visualize the DNA localization by photography on a UV transilluminator.

In Vitro Transfection Efficiency

L929 mouse fibroblasts (DSMZ, Braunschweig, Germany) were plated 24 h before nanoparticle incubation at a concentration of 50,000 cells / 2 ml in DMEM medium supplemented with 10% fetal calf serum (FCS) in 12 well plates. Immediately prior to transfection, the medium was removed and replaced by fresh DMEM containing 10% FCS.

Aliquots of the microparticle suspension containing 4 µg pLuc-CMV were added to the medium. The cell culture medium was removed after 4 hours and replaced with fresh medium containing 10% FCS. Cells were harvested after 48 h and washed with PBS pH 7.4 twice, and lysed in cell culture lysis reagent (Promega, Mannheim, Germany). Luciferase content was assessed using a commercial luminescence kit (Promega) and a Berthold Sirius luminometer (Berthold, Pforzheim, Germany). RLUs were converted into luciferase content by calibration with recombinant luciferase (Promega). Protein concentrations were determined by a modified BCA assay [9]. Transfection experiments were performed in triplicate and presented as the mean of the luciferase / protein ratio [ng/mg].

Cellular Uptake of DNA Nanoparticles

For confocal microscopy experiments, a Zeiss Axiovert 100M microscope coupled to a Zeiss LSM 510 scan module was used. Plasmid DNA was fluorescently labeled with a Cy-3 rhodamin dye (Mirus, Madison, Wisconsin) according to the manufacturers instructions.

The RG 502H+PEI 10% microparticles were fluorescently labeled by covalent coupling with Oregon green 488 (Molecular Probes, Leiden, The Netherlands). Briefly, the dry particles without lyoprotectant were re-suspended in 1 ml ultrapure water at pH 8. 10 µl of Oregon green in DMSO was added to the suspension and stirred for one hour with the particles at room temperature. The suspension was centrifuged 10 min at 3000 rpm in a 5415C Eppendorf centrifuge at 4°C and washed 4 times with ultrapure water. The resuspended particles were freeze-dried and stored at –20°C until further use.

DNA was adsorbed according to the conditions used for the transfection assays. Briefly, Cy-3 labeled DNA was mixed with the original DNA (1:1) and was incubated for one hour with the Oregon green labeled particle suspension at a DNA / particle ratio of 1:100 [$^{m}/_{m}$].

L929 cells were seeded at a density of 20,000 cells per well in 8 well chamber slides (Lab Tek, Nunc, Wiesbaden, Germany). After 24 hours the medium was removed. Aliquots of the resulting nanoparticle suspension containing 0.8 µg DNA were added to new medium containing 10% FCS. The cells were incubated with the nanoparticles for 5, 15, 30, 60 and 180 minutes. The medium was removed and cells were washed 4 times with PBS buffer. Fixation of cells was performed by incubation with 400 µl paraformaldehyde solution 3% in PBS for 20 minutes. The cells were washed 4 times with PBS and incubated for additional 20 minutes with a 0.1 mg/ml DAPI (4',6-diamidino-2-phenylindole, dihydrochloride, Molecular Probes, Leiden, The Netherlands) solution in PBS for nucleus staining. For excitation of the blue DAPI fluorescence an Enterprise UV laser with an excitation wavelength 364 nm was used. Excitation of the green fluorescence of 5-DTAF labeled polymer was obtained using an argon laser with an excitation wavelength of 488 nm and for excitation of red fluorescence of the DNA a Helium-Neon laser with an excitation wavelength of 543 nm was used. Images were recorded in multitracking mode using a longpass filter of 385 nm for DAPI, a longpass filter of 505 nm for Oregon Green and a longpass filter of 560 nm for rhodamine.

In Vivo Immunization

Female BALB/c mice (6-8 week-old) were purchased from the Federal Institute for Risk Assessment, Berlin, Germany and maintained under specific-pathogen-free conditions in the animal facilities of the Federal Institute for Risk Assessment, Berlin, Germany, or in the animal facilities of the Max-Planck-Institute for Infection Biology, Berlin, Germany. All animal experiments were performed in accordance with German and institutional animal care guidelines.

Listeria monocytogenes EGD strain Sv 1/2a, a laboratory wild-type strain was originally obtained from G. B. Mackaness. The bacteria were grown in Luria-Bertani (LB) broth (Difco, Heidelberg, Germany) without any antibiotics to an

OD_{600} of 0.6, harvested by centrifugation, and stored as stock in final 10% glycerol in LB at –80ºC. The next day, one stock was thawed, plated onto LB agar plates, and colony-forming units (CFU) were assessed.

Sex- and age-matched BALB/c mice in groups of six mice were immunized with 10 or 100 μg of naked DNA or with 10 μg of DNA adsorbed on 1 mg of the microparticle formulation. Immunizations were performed 3 times at 3 weeks intervals by intramuscularly (i.m.) injection of 100μl. Microparticles were prepared under aseptic conditions. The freeze dried particles were re-dispersed with distilled water and incubated with the DNA constructs over 12 hours at 4°C.The vaccination protocol was optimized by Fensterle et al.. As positive control, sublethal dose ($0.1XLD_{50}$) of *L. monocytogenes* EGD strain was injected intravenously (i.v.) into mice at the same time as the prime vaccination. Mice vaccinated with DNA encoding *L. monocytogenes* genes were challenged i.v. with lethal dose ($5XLD_{50}$ or $10XLD_{50}$) of *L. monocytogenes* strain EGD in 100μl of sterile PBS, at day 0, 3 weeks after the last boost. Survival was checked daily until day 10 post infection.

RESULTS AND DISCUSSION

Multiple strategies of adjuvant systems have been investigated for the effective use of DNA vaccines. One of them represents particulate systems, which have been intensively studied by several groups [3,10,11]. The overall aim of vaccinologists using particulate systems has been to obtain antigen presentation via the (major histocompatibility) MHC I pathway, providing new possibilities to act against intracellular pathogens and tumors. With this objective, diversified processes for the formation of biodegradable microparticles were studied, such as the encapsulation of DNA by modified double emulsion methods [11], spray-drying or the adsorption of the DNA on cationic microparticles [3,12]. It has

become clear that the encapsulation and further a controlled release of the large and hydrophilic DNA in a bioactive form was a delicate ambition [13]. Moreover, the influences of the release kinetics of DNA from the microparticles on the immune response have not yet been fully elucidated.

However, recently it was shown that the reduction of the interval between the emergency of the danger signal, induced by the injection of the particulate matter, and DNA release is crucial for the induction of an immune response [14]. T achieve this, we developed a new type of cationic microparticles by incorporation of PEI 25 kDa into the biodegradable polymeric matrix. PEI 25 kDa is a well known and highly efficient DNA transfection agent [5]. These microparticles were prepared by a modified double emulsion procedure. A summary of microparticle characteristics is shown in Table 1. ξ-potential measurements were performed to evaluate the capability of DNA adsorption via ionic interactions on the microparticle surfaces.

This study demonstrated that only the incorporation of PEI into the polymer was able to produce positive surface charges.

Particles prepared with the plain polyester RG 502H, using CTAB in the external phase had low positive ξ-potentials. RG 505 polymer particles exhibited a slight negative ξ-potential, when prepared in CTAB. The ξ-potentials of particles prepared with PVA as an external stabilizer were negative, irrespective of the PLGA used. The blending of the polyester matrix with increasing concentrations of PEI led to the reversal of charge from - 22.9 mV to + 47.3 mV. For example, particles prepared with 1 % PEI still had a negative ξ-potential which reversed to positive values when particles were prepared with a 5 % PEI content.

The incorporation of PEI into the polymer matrix was possible due to the solubility of PEI in methylene chloride, the solvent used for microparticle formulation. A partition coefficient of 2.9 : 1 (water : methylene chloride) of PEI 25kDa was determined in the two solvents, water and methylene chloride.

Polymer	Cation	Stabilizer[a]	Size [µm]	ξ - Potential [mV]	Efficiency [%][b]
RG 505		CTAB	37.6 ± 39.9	- 4.40 ± 0.4	33.7 ± 9.5
		PVA	3.29 ± 3.2	- 24.6 ± 0.7	1.53 ± 2.2
RG 502H		CTAB	30.5 ± 14.2	5.23 ± 0.1	11.3 ± 2.5
		PVA	3.82 ± 0.8	- 16.7 ± 0.5	12.0 ± 9.8
	PEI 0.1%	PVA	13.4 ± 1.0	- 22.9 ± 1.0	16.0 ± 1.6
	PEI 0.5%	PVA	0.93 ± 0.2	- 23.0 ± 0.3	18.1 ± 3.1
	PEI 1%	PVA	17.4 ± 9.0	- 17.2 ± 0.8	10.4 ± 1.8
	PEI 5%	PVA	6.94 ± 1.3	17.0 ± 2.8	10.9 ± 1.0
	PEI 10%	PVA	1.39 ± 0.2	47.3 ± 1.2	96.3 ± 4.7
	PEI 10%	CTAB	1.44 ± 0.6	17.7 ± 0.5	96.7 ± 4.0
	PEI 50%	PVA	15.2 ± 2.1	39.2 ± 0.7	31.3 ± 1.5
	CTAB 0.1%	PVA	17.1 ± 3.8	- 22.7 ± 0.3	18.7 ± 13.1
	CTAB 1%	PVA	22.0 ± 1.4	- 19.0 ± 1.5	18.2 ± 13.6
	CTAB 10%	PVA	56.0 ± 15.1	- 13.9 ± 0.8	13.0 ± 1.9
	CTAB 50%	PVA	63.1 ± 10.7	- 14.7 ± 0.3	24.1 ± 6.9

Table 1: *Characterization of microparticles prepared by blending PLGA with cationic components.*

[a] 0.5% PVA or CTAB in distilled water.

[b] DNA loading efficiency using a 0.5 mg/ml DNA solution in distilled water for incubation with the microparticles suspended in distilled water with a resulting theoretical DNA loading of 1%.

Thus, a diffusion of the cationic agent from the methylene chloride solution into the aqueous stabilizer solution was expected. This, however, did not result in the complete redistribution of PEI into the external aqueous phase, as demonstrated by the highly positive ξ-potential of microparticles prepared with 10% PEI.

Another cationic agent, CTAB, was used to prepare microparticles with the aim to create a cationic surface for DNA adsorption. In contrast to the PEI blend particles, these microparticles did not exhibit positive ξ-potentials. Blending PLGA with CTAB in concentrations from 0.1 % to 50 % only led to an increase in the ξ-potential of only –22.7 to –14.7 mV. This could possibly be explained by a different arrangement of the cationic molecule in the biodegradable polymer matrix. CTAB was soluble in methylene chloride to some extent. The partition coefficient of CTAB in methylene chloride and the aqueous solution was determined to be 1 : 2.32 (water : methylene chloride). We assumed, that CTAB induced the formation of reversed micelles when the aqueous medium (PBS buffer) was added to the organic solution [15]. In consequence CTAB would have accumulated in the water/methylene chloride interphase, orienting the polar head group into the core of the micelle. This is a reasonable assumption, since the CMC of CTAB (21.1 mg/ml) was exceeded in the formulation. Taking into account the highly negative ξ-potentials of the CTAB microparticles, a subsequent rearrangement of the detergent did not occur. This explanation of the ξ-potential values was further supported by the shift of ξ-potential towards higher values for the particles containing increasing amounts of PEI, whereas the increase of the amount of CTAB in the formulation only had a minor effect on the ξ-potential. Still, the cationic charge density of the two agents has to be considered, as PEI has a very high amine density, compared to CTAB which contains only one permanent positive charge per molecule.

Microparticle sizes ranged from 63.05 µm to 0.93 µm, depending on the external stabilizer and the cationic excipient used during particle preparation. CTAB exhibited an important influence on the microparticle size when used

both in the external stabilizer solution or when added to the internal phase. Both formulations with CTAB (RG 505, RG 502H) as cationic stabilizer showed approximately 10-times larger hydrodynamic diameters than the analogous preparations in PVA solution. The type of PLGA (RG 505, RG 502H) had no effect on the particle size. All subsequently prepared microparticles were formulated using the lower molecular weight and end-group un-capped PLGA (RG 502H), due to its faster degradation characteristics compared to the high molecular weight RG 505 polymer [16]. Increasing amounts of CTAB added to the polymer solution in methylene chloride and PBS buffer resulted in a substantial increase in particle size of the microparticles. Increasing amounts of PEI in the PLGA polymer, however, did not have any effect on the final particle size. The considerable increase in size of the CTAB containing microparticles (0.1% – 50%) can be ascribed to the surface active properties of the cationic agent, acting as plasticizer within the polymer matrix. This, in consequence, resulted in the aggregation of the microparticles during their preparation or during their isolation. This hypothesis was reinforced by the 10-fold larger particle diameter of microparticles prepared in CTAB solution compared to those formulated in PVA by the same procedure.

The adsorption efficiency of DNA was investigated in water at pH 7 in presence of the sucrose used for lyophilization. The DNA adsorption onto microparticles exhibiting negative ξ-potentials was probably the result of non-ionic interactions. In contrast, the adsorption efficiencies of microparticles prepared with the 10% PEI blend with PLGA correlated with the extremely high ξ-potential of these formulations, resulting in an approximately 100% DNA adsorption efficiency. However, the microparticles formulated with 50% PEI exhibited a reduced adsorption efficiency compared to the PEI 10% preparation. Under these conditions, PEI could possibly be detached from the PLGA matrix, causing the lower adsorption efficiency.

Interestingly, an increased DNA adsorption efficiency was measured for the RG 505 / CTAB preparation compared to the analogue preparation in PVA. The 50% CTAB blend preparation, as well showed higher DNA adsorption. The ξ-potentials of these particles were demonstrated to be negative, therefore, an additional factor must have influenced the DNA / microparticle interaction, possibly the large size of the particles and their aggregated structure. An efficient DNA adsorption of the RG 505 / CTAB preparation has already been demonstrated by others [3]. With the preparation methods used in this study, we did not realize efficiencies as high as those reported, but we did detect adsorption of DNA onto the particles. No difference in adsorption efficiency was seen for the RG 502H set of particles either prepared with PVA or CTAB as a stabilizer.

SEM micrographs of the microparticles, RG 502H +10% PEI, prepared in either PVA or CTAB and microparticles prepared with CTAB, either in the external phase or internalized, confirmed the PCS data (Fig.1).

Interestingly, multiple pores in the particle surface could be observed for both +10% PEI preparations, suggesting that adsorption was improved by the larger surface area available. Particles prepared with the detergent CTAB were all highly aggregated. The RG 505 microparticles formulated with 0.5% CTAB in the external phase showed small, but highly aggregated particles. Those in which CTAB had been incorporated in an amount of 10% and 50% exhibited larger agglomerates. This finding was consistent with the size measurements. The CTAB micrographs showed that CTAB was responsible for aggregation as it is able to integrate in the polymer surface. The microparticles with 50% PEI did not show particles of regular shape. In this formulation, the amount of water soluble component disrupted particle formation.

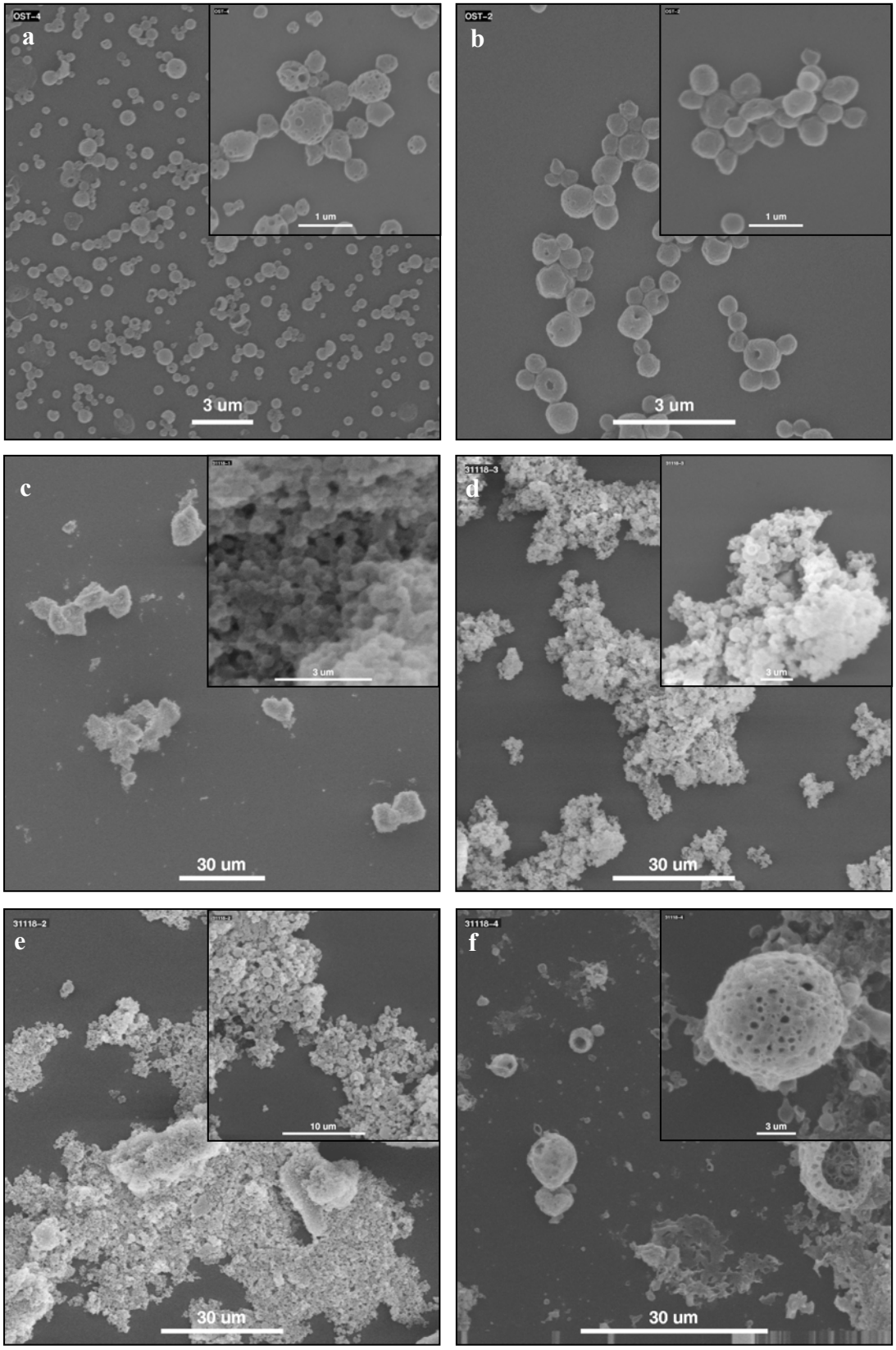

Fig.1: *SEM micrographs of the particles, RG 502H+PEI 10% in 0.5%PVA (a), RG 502H+PEI 10% in 0.5% CTAB (b), RG 505 in 0.5% CTAB (c), RG 502H+CTAB 10% in 0.1% PVA (d), RG 502H+ CTAB 50% (e), RG 502H+PVA 50% (f).*

Specific microparticles were chosen for further analysis. The DNA adsorption, as well as the ξ-potential in low ionic strength buffers with different pH (Fig.2) were investigated. Low ionic strength buffers were chosen to reduce the influence of buffer components on the ξ-potential measurement [17]. We intended to evaluate the surface charges at different degrees of protonation and possibly correlate them with DNA adsorption characteristics. The formulations chosen were RG 505 / CTAB, RG 502H / PVA and the highly adsorbing RG 502H + 10% PEI prepared in either CTAB or PVA. The RG 505 / CTAB, as well as RG 502H / PVA particles displayed negative ξ-potentials over the pH range from 8 to 5. In contrast, the microparticles formulated with RG 502H + PEI 10% blends exhibited positive ξ-potentials over the full pH range from 3 to 8. For the latter particles, CTAB stabilization led to higher values than those stabilized with PVA.

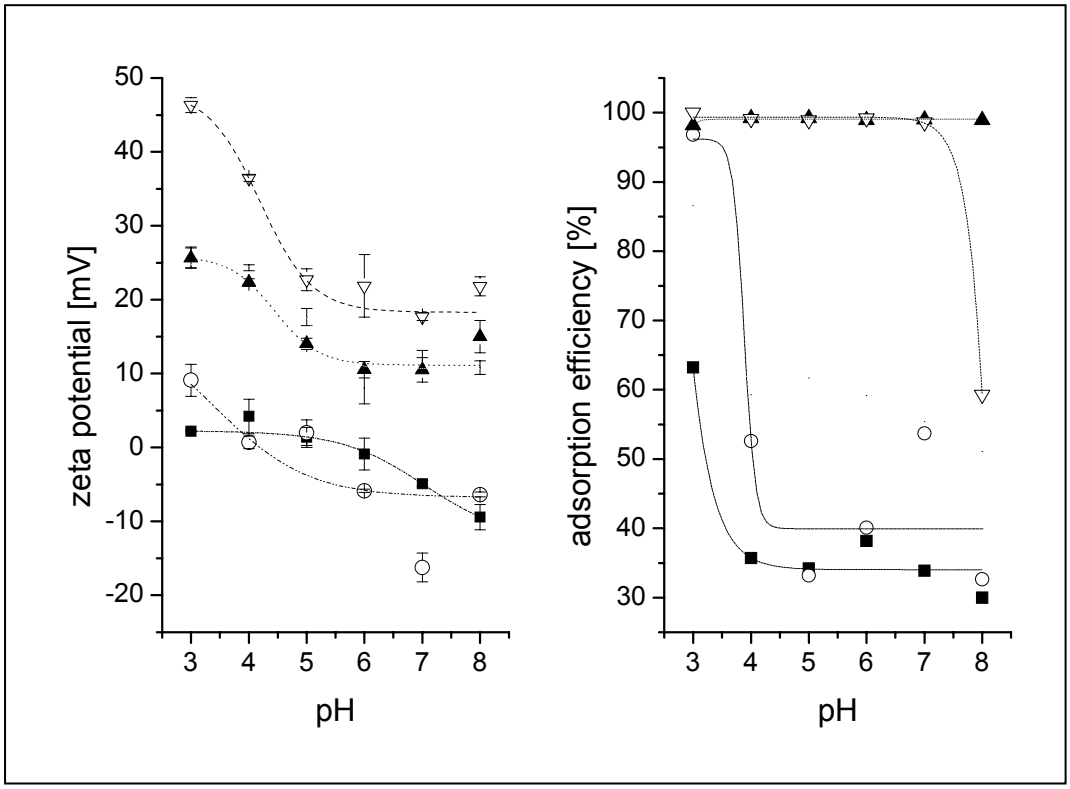

Fig.2: *ξ – potential and adsorption efficiency of microparticles in low ionic buffer (I=0.01) at pH 3 – 8. RG 502H / PVA (■), RG 505 / CTAB (○), RG 502H+PEI / PVA (▲), RG 502H + PEI / CTAB (▽).*

The pronounced increase in ξ-potentials seen for PEI blend microparticles demonstrated the presence of protonable groups on the surface of the particles. This explanation was reinforced by negligible changes in the ξ-potential of particles formulated without PEI (RG 502H / PVA).

There are two possible reasons for the increased ξ-potential of the CTAB stabilized RG 502H+PEI particles. Either CTAB was integrated within the polymer surface or the increase was due to the absence of PVA interaction with the polymer surface. PVA is known to be to some extent associated with the particle surface during particle preparation [18]. Therefore, PVA stabilized PLGA particles usually exhibited negative ξ-potentials, as demonstrated for the RG 502H / PVA formulation. Since the CTAB stabilized preparations did not exhibit greatly increased ξ-potentials, we concluded that the increased ξ-potentials of the 10% PEI blend particles in CTAB arose from the absence of PVA stabilizer, rather than the presence of CTAB.

The ξ-potentials correlated well with the DNA adsorption efficiencies, which were measured in the same low ionic strength buffers from pH 3 to pH 8. Both microparticle formulations containing PEI, either prepared in PVA or CTAB exhibited almost complete DNA adsorption efficiencies over the investigated pH range. The DNA adsorption onto RG 505 / CTAB and RG 502H / PVA particles increased in the acidic environment only, from pH 4 onwards.

The membrane toxicity of the cationic microparticles and cationic agents were investigated by LDH release from L929 mouse fibroblasts in vitro (Fig.3). Cationic agents have often been demonstrated to induce membrane toxicity, due to electrostatic interactions with negatively charged glycocalyx of the cellular surface [19,20]. The levels of LDH release obtained for microparticles prepared with increasing amounts of PEI 25 kDa, were, with the exception of the 50% PEI formulation, less than 10%, the level at which preparations are considered to be toxic [19]. Despite the high ξ-potential of the PEI 10% formulation no membrane toxicity was observed.

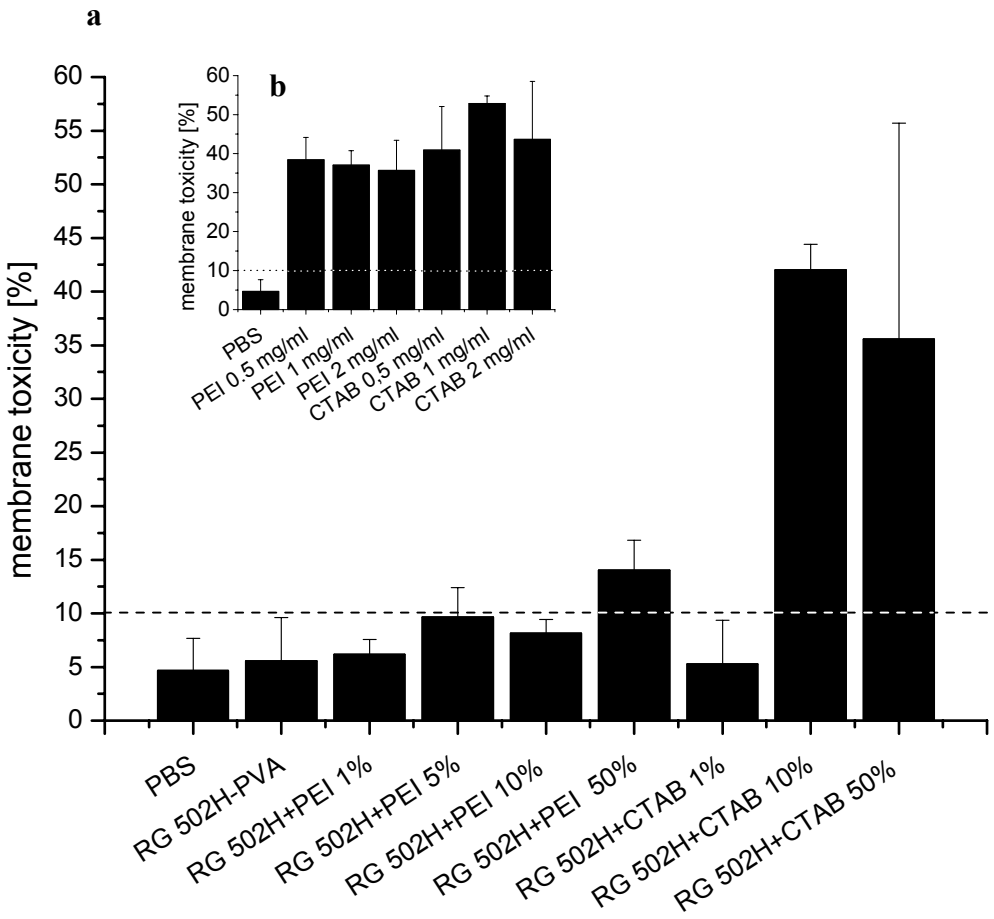

***Fig.3**: Membrane toxicity analyzed by LDH release from L929 mouse fibroblasts after 3 hours of incubation with microparticles (a) [1 mg/ml] and (b) CTAB and PEI 25kDa in solution.*

This effect was explained by the incorporation of PEI into the PLGA matrix reducing the interactions with the cellular membrane and the accessibility of the charges. The PEI 50% blend formulation exhibited higher and toxic LDH release, possibly arising from the rapid disintegration of the particle resulting in PEI 25kDa release. Interestingly, microparticles prepared with 10% or more CTAB showed extensive toxicity levels, even though these preparations exhibited negative ζ-potentials. These findings can be explained by the dissociation of CTAB from the formulation and the detergent effect of CTAB. The effects of the microparticle formulations on membrane stability were

compared to the effects of the cationic agents in solution. Three concentrations of PEI and CTAB were incubated with fibroblasts, resulting all cases in high LDH release levels. As a consequence, both cationic agents, PEI 25 kDa and CTAB, were deemed toxic to cellular membranes.

The stabilizing effect of cationic microparticles on DNA degradation by DNase was investigated by agarose gel electrophoresis (Fig.4). DNA integrity was analyzed by the application of an emulsion, using a phenol / glycerol mixture with TBE buffer containing the dissolved formulations after the incubation with the enzyme. As a result, no DNA extraction was necessary from the particle formulations after the incubation with the enzyme. Naked DNA was degraded within the first five minutes of incubation with DNase I (Fig.4a). The influence of the two cationic agents was investigated in Figure 4b/c.

CTAB exhibited a stabilizing effect on DNA, however, DNA was completely degraded after 20 min of enzyme incubation. In contrast, PEI 25 kDa protected DNA against degradation over a 12 hour time period, although a conversion of the supercoiled to the open circular form was observed. This highlighted the excellent DNA condensation capabilities of PEI 25kDa. CTAB, as a single tailed cationic lipid, has been used for plasmid DNA isolation by precipitation, thus the CTAB / DNA interaction was expected to result in enzyme stabilization [4]. However, the protection against DNase I degradation was relatively low. This was attributed to the low electrostatic interactions of the single charged molecule, used at a 1:1 [$^{m}/_{m}$] ratio with DNA, compared to polycations.

PEI containing microparticles, which displayed negative ξ-potentials and, as a result low adsorption efficiencies, for example RG 502H+PEI 1% did not protect DNA from enzyme degradation (Fig.4d). DNA was degraded within 5 minutes of incubation. However, microparticles formulated using higher amounts of PEI, such as RG 502H+10% and +50%, (Fig.4e/f) protected the adsorbed DNA over almost 12 hours. Similar to DNA/PEI complexes, DNA exhibited a change to the open circular form in later time points of incubation.

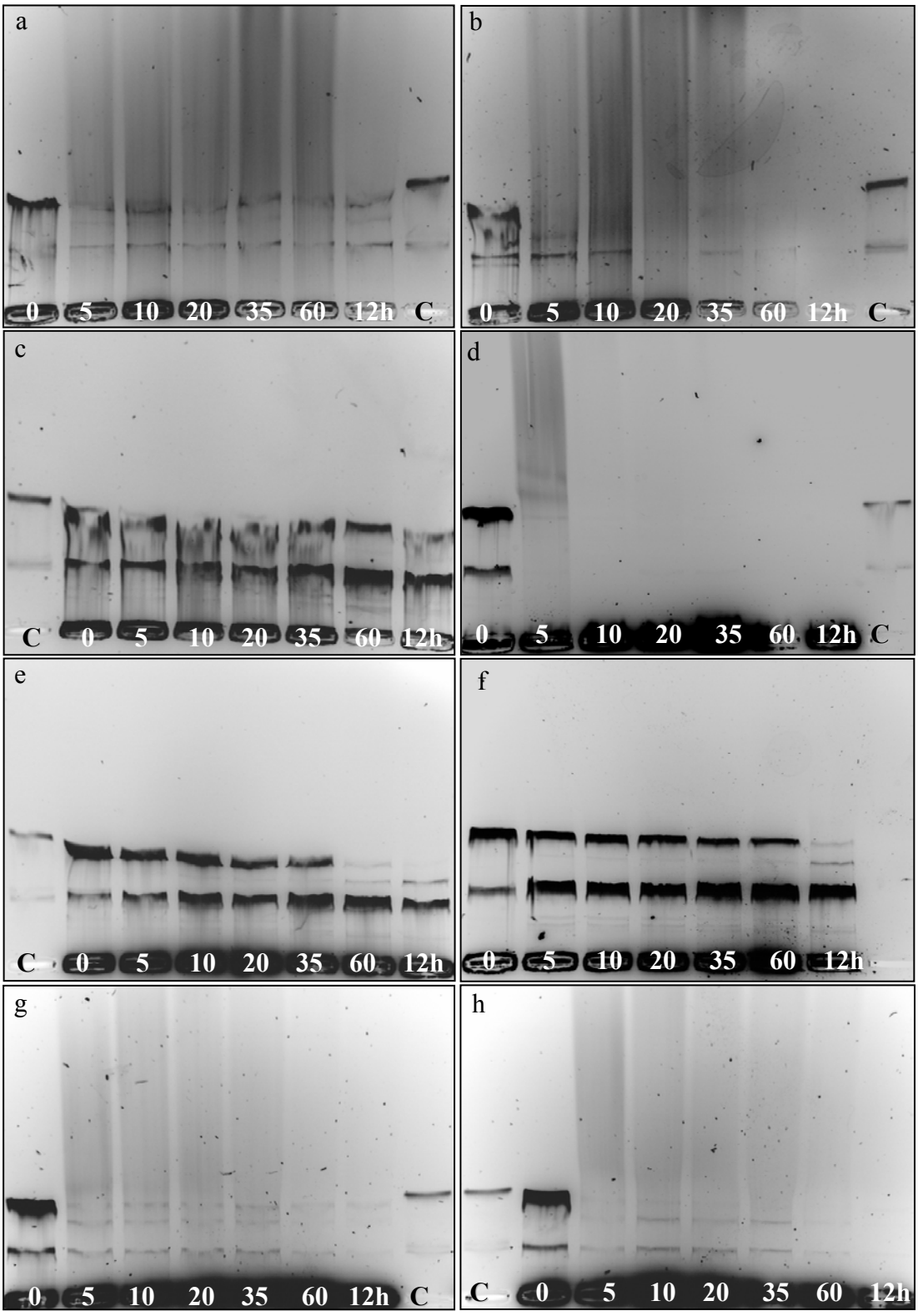

Fig.4: *DNA stabilization against DNase degradation, (a) DNA, (b) DNA/CTAB, (c) DNA/PEI, (d) DNA adsorbed on RG 502H+PEI 1%, (e) DNA adsorbed on RG 502H+PEI 10%, (f) DNA adsorbed on RG 502H+PEI 50%, (g) RG 502H+CTAB 10%, (h) RG 502H+CTAB 50%.*

We attributed the high protection efficiency of the RG 502H+PEI 50% formulation to the formation of DNA polyplexes, as PEI appeared to be only loosely associated with the PLGA polymer. The RG 502H+PEI 10% formulation showed considerable DNA stabilization in agreement with the high ξ-potential and the nearly complete adsorption of 1% [$^{m}/_{m}$] DNA. This finding was not attributed to a polyplex formation, since the low membrane toxicity suggests the absence of free PEI. The protection of DNA microparticles has so far only been demonstrated for DNA encapsulated in PLGA particles as well as for DNA adsorbed on aminosilanes modified silica nanoparticles and mineral surfaces [21-23]. In contrast, none of the microparticles prepared with CTAB displayed a stabilizing effect on DNA (Fig.4g/h). In both preparations formulated either using 10% or 50% CTAB the DNA was degraded after only 5 minutes of incubation, demonstrating the inefficient adsorption.

Transfection experiments were carried out with plasmid DNA microparticles incubated in a 1: 100 [$^{m}/_{m}$] ratio, corresponding to a DNA loading of 1% (Fig.5). The transfection efficiencies were relatively high for particles prepared by the incorporation of 10% and 50% PEI, when compared to DNA/PEI complexes at a N/P ratio of 5 (Fig.5a). The mechanism of PEI mediated gene delivery has been demonstrated to be based on the osmotic rupture of endosomes, resulting in the release of DNA into the cytosol [5,24,25]. However, the non-phagocytic cells used in this study could not take up the microparticles. Thus, another mechanism of gene delivery must have occurred. The blend particles with a lower content of PEI did not lead to effective gene delivery. Likewise, the formulations with CTAB and the DNA / CTAB 1/1 [$^{m}/_{m}$] mixture did not result in a significant transfection efficiency in vitro. These results are in line with observations made previously, showing that complexes of plasmid DNA with CTAB do not have a significant influence on DNA delivery and transfection [26]. Microparticles prepared with 10% PEI were further investigated, by varying the theoretical DNA loading.

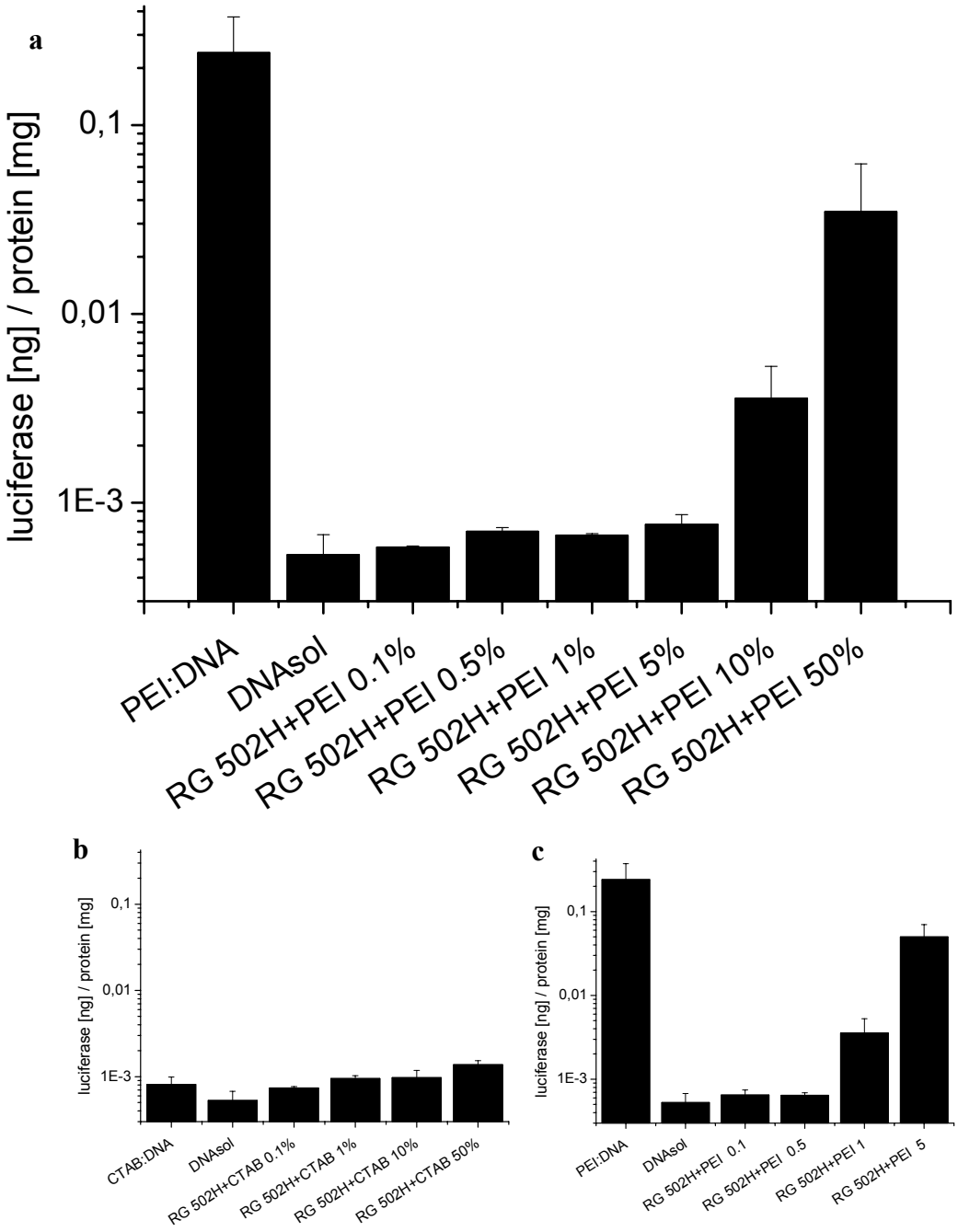

Fig.5: *Transfection efficiency of DNA microparticles. (a) Transfection of DNA adsorbed onto PEI blend RG 502H microparticles compared to the efficiency of DNA/PEI complexes at N/P 5. Microparticles with 10% and 50% PEI exhibited efficient gene delivery. (b) Transfection of DNA adsorbed onto CTAB blend particles compared to a DNA / CTAB 1/1 [$^{m}/_{m}$] preparation. (c) Transfection of luciferase DNA using different theoretical loadings on RG 502H+PEI 10%.*

This was performed using constant amounts of DNA with increasing amounts of particles (Fig.5c). Higher amounts of particles mixed with constant amounts of DNA led to higher transfection efficiencies. The transfection of non-phagocytic cells, using cationic DNA microparticles exceeding the cutoff size of 0.5 µm for endocytotic uptake, has been discussed recently [26,27]. In these previous studies, the mechanism of gene transfer was ascribed to the detachment of the cationic agent from the microparticle, resulting in a polyplex transfection. However, the transfection mediated by covalently attached PEI 25 kDa on microspheres or polymer films has been described by Zheng et al. [28]. In this case, the transfection was attributed to a pH independent membrane disruptive effect [29].

This group also conceded the possibility of enzyme cleavage of the linker carrying PEI. From our data, we were not able to detect increased rates of membrane disruption. This was demonstrated by the low LDH release from cells incubated with the particles formulated with PEI blends up to 10%. Therefore, we hypothesized that the transfection was mediated by the physical approach of DNA loaded microparticles towards the cell surface, as suggested by Ogris et al. [30]. This facilitated the endocytosis of either naked DNA or detached DNA/PEI complexes.

To study the transfection mechanism of DNA loaded microparticles, both PEI and DNA were fluorescently labeled. Non-phagocytic L929 cells were incubated with DNA microparticles for 3 hours and fixed. The confocal microscopy revealed high concentrations of the formulations on the surface of the cells. The colocalization of both covalently bound fluorescence markers demonstrated the conservation of the PEI/DNA complexation during incubation. The cells exhibited a diffuse green and red fluorescence throughout the cytosol in combination with some concentrated fluorescence. These observations could be explained by the membrane disruptive properties of high concentrations of PEI/DNA complexes on the surface of the cellular membrane [29]. Segura et at

al. achieved similar transfection levels weather using biotinylated PEI bound to neutravidin, which was covalently bound to the cell culture dish, or weather non-biotinylated PEI was nonspecifically adsorbed to that surface [31]. However, in this study 20% of PEI, specifically-bound or nonspecifically-adsorbed, was released within 2 days. Therefore both transfection results could arise from un-bound DNA/PEI complexes. In our study the diffuse fluorescence of both the PEI label and DNA label showed that this complex is released into the cell, possibly through small local damages in the cellular membrane. This mechanism, however, has to be further investigated.

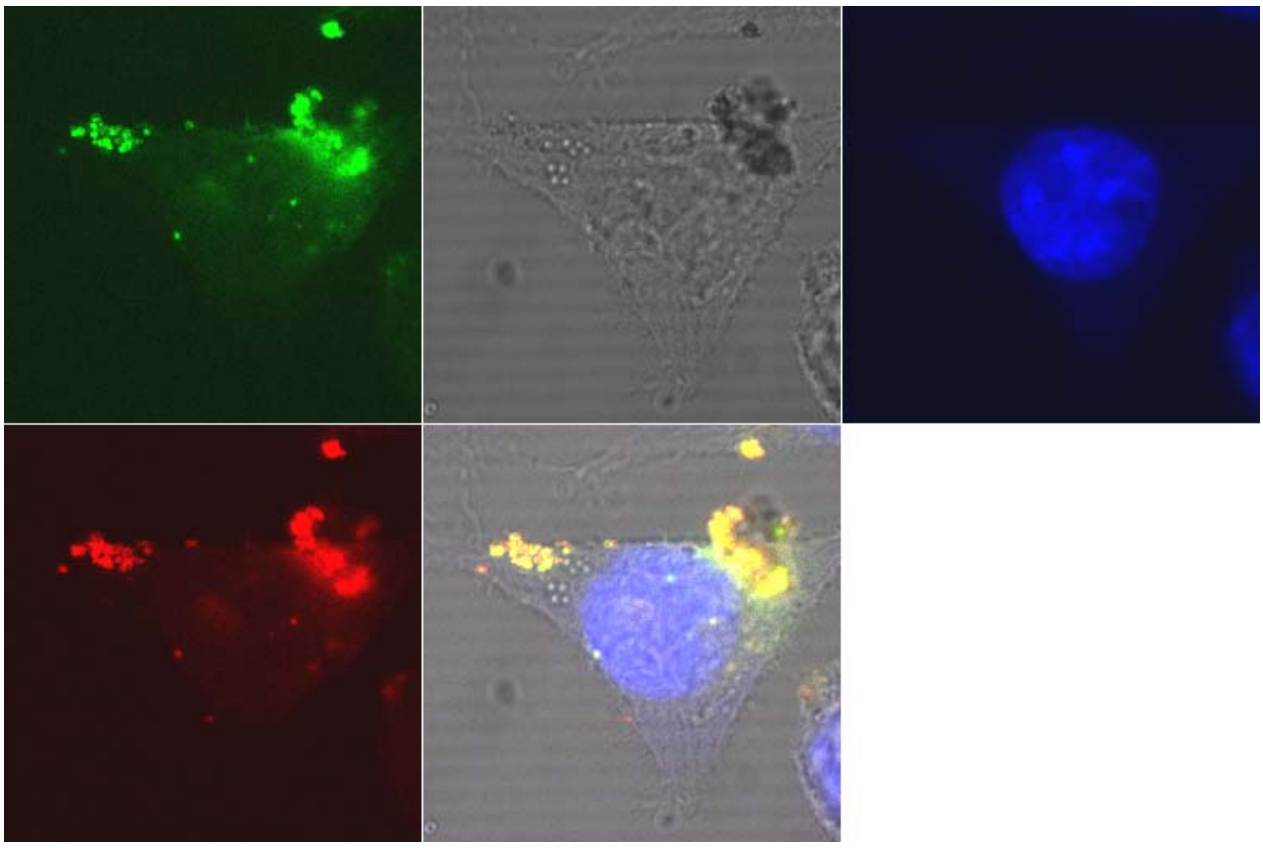

Fig.6: *Confocal microscopy of L929 mouse fibroblasts after 3 hours of incubation with Oregon green labeled RG 502H+PEI 10% (green) with Cy-3 labeled DNA (red). The nucleus was stained using DAPI (blue). The DNA and PEI are co-localized. High concentrations of the microparticles were adsorbed on the cell. A diffuse fluorescence in the cell indicated that PEI and DNA were taken up into the cell.*

The protection of mice immunized with DNA against antigens of *Listeria monocytogenes* adsorbed onto cationic microparticles was studied by measuring their survival after a lethal challenge. *Listeria monocytogenes* is an intracellular bacterium. Hence, a successful immunization must be T-cell mediated to eliminate infected cells. The survival rates of mice challenged with lethal doses of *Listeria monocytogenes* are represented in Figures 7 and 8. In the first setting (Fig.7) both groups of mice were either immunized with 10 µg naked DNA or 10 µg DNA adsorbed onto RG 502H+PEI 10% microparticles.

The antigen encoding vectors of Fensterle et al. were used [6]. After one prime and two booster immunizations the mice were challenged with $5xLD_{50}$ *Listeria monocytogenes*. Naïve mice of both groups and mice immunized with the p60 encoding sequence died within 4 to 7 days from the Listeria infection. All mice actively immunized with the sublethal dose of *Listeria monocytogenes* survived the challenge in both groups. A significant difference was observed for the survival of mice immunized with a sublethal dose of *L. monocytogenes* and mice immunized with the vector control, referred to as 'mock' DNA adsorbed onto the microparticles (P=0.0178) (Fig.7a).

This mock DNA, which did not encode a Listeria antigen was capable of protecting 33% of the mice encoding the typical listeriolysin, and the mutant LLO vector, led to higher survival rates compared to the naked DNA.

Mice immunized with mutant LLO DNA adsorbed onto microparticles exhibited the highest survival rates. In this group 66% (4 out of 6) mice survived. The effect of DNA adsorption on survival, however, was not significant compared to that of mock (P=0.2029).

Therefore, the experiment was repeated with higher dose of *L. monocytogenes* (Fig.8). In this experiment mice were either immunized with 100 µg naked DNA (Fig 8b) or the mice were immunized with 10 µg DNA adsorbed onto microparticles (Fig.8a) using $10xLD_{50}$ *L. monocytogenes* to challenge the mice.

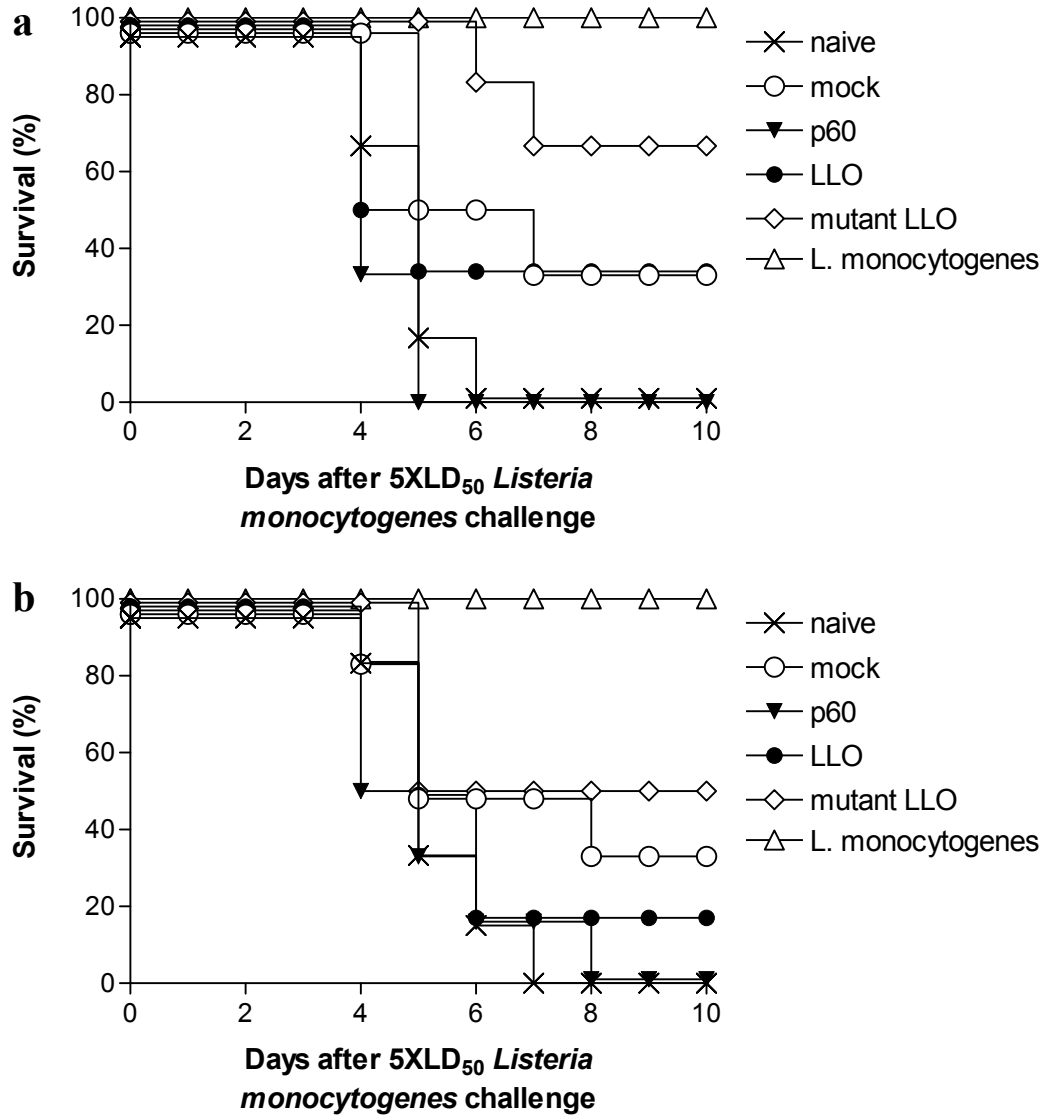

Fig.7: *Survival curves of balb-c mice. (a) Intramuscular immunization with 10 µg DNA adsorbed onto 1 mg microparticles. The difference of survival of mice immunized with mutant LLO was not significant compared to the mock (P=0.2029). Difference of survival of mice immunized with a sublethal dose of L. monocytogenes was significant compared to that of mock (P=0.0178). (b) Intramuscular immunization with 10 µg naked DNA. The difference of survival with mutant LLO was not statistically significant compared to mock (P=0.5143). The difference of survival of mice immunized with sublethal dose of L. monocytogenes was significant compared to that of mock (P=0.0185). The difference of survival of mice immunized with mutant LLO adsorbed on microparticles was not significant compared to naked mutant LLO (P=0.4441). Statistics was assessed by logrank test using Prism software.*

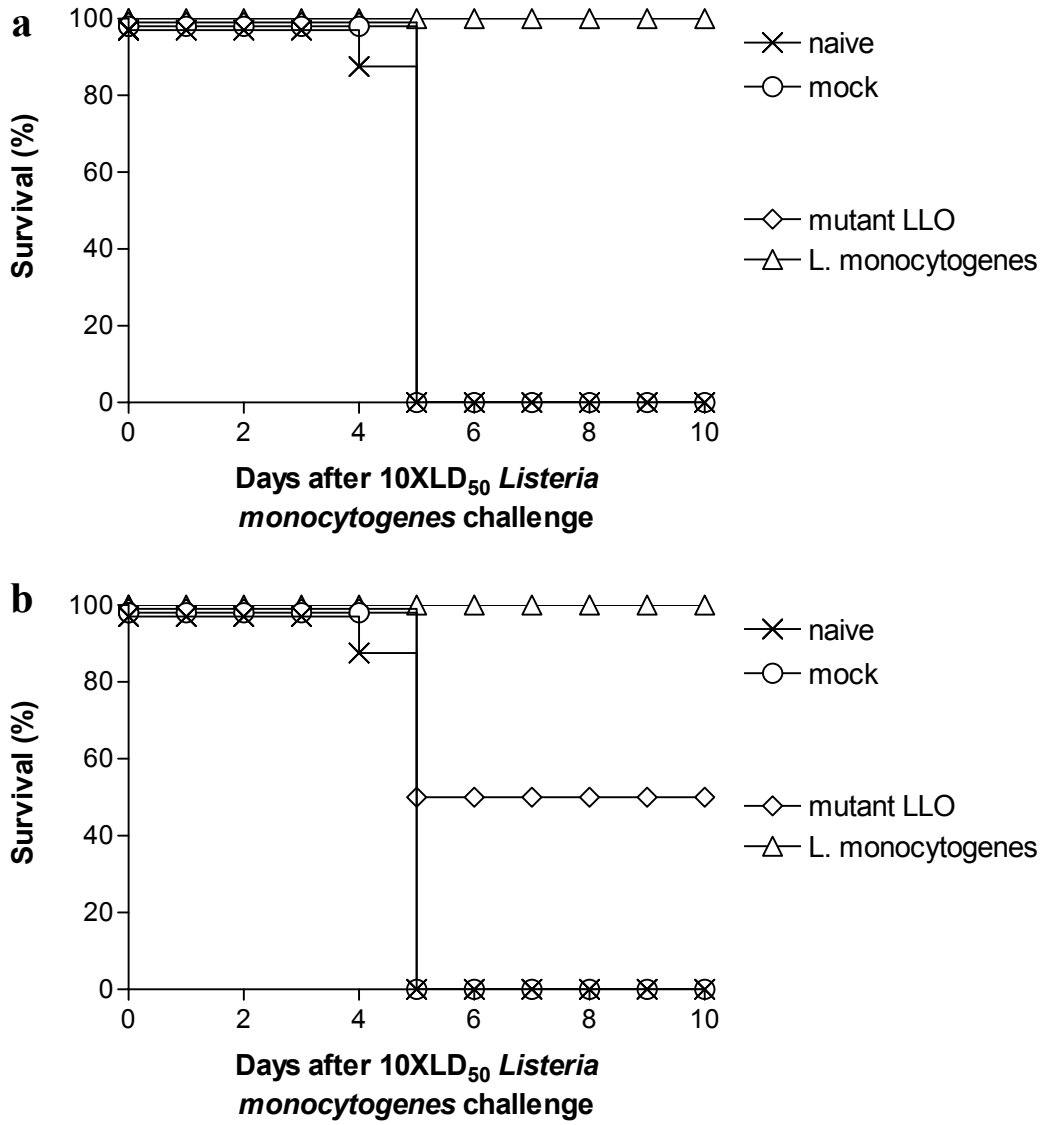

Fig.8: *Survival curves of balb-c mice. (a) Intramuscular immunization with 10 µg DNA adsorbed onto 1 mg RG 502H + PEI 10% microparticles. (b) Intramuscular immunization with 100 µg naked DNA. The difference of survival of mice immunized with mutant LLO was not statistically significant compared to that of mock (P=0.0701). The difference of survival of mice immunized with sublethal dose of L. monocytogenes was significant compared with that of mock or mutant LLO (P=0.0498). Statistics was assessed by logrank test using Prism software.*

The purpose of this study was not only to improve the protection but also to reduce the amount of DNA required for efficient vaccination. All but the actively immunized mice died in the DNA microparticle group. The immunization with 100 μg naked DNA (mutant LLO), however, could protect 50% of the mice. The difference of survival of mice immunized with naked mutant LLO was not statistically significant compared to that of naked mock (P=0.0701). Still, the difference of survival of mice immunized with sublethal dose of *L. monocytogenes* was significant compared with that of naked mock or mutant LLO (P=0.0498). Therefore, we could conclude that the adsorption of DNA onto RG 502H+PEI 10% microparticles could induced higher survival rates after a lethal challenge when equal doses of DNA were used for immunization, even if these effects were not equal to a 10-times higher dose of naked DNA.

CONCLUSION

In this study we developed a cationic microparticulate system by the incorporation of the cationic molecules, PEI or CTAB, into the polyester matrix. PEI 25kDa, added to the polymer at 10% exhibited the most promising characteristics of all microparticle formulations.

The adsorption efficiency was complete for a theoretical loading of 1% over a pH range from 3 to 8. The ζ-potential was + 47.3 mV and, thus, correlated with the adsorption efficiency. Further, this formulation protected adsorbed DNA from enzyme degradation over 12 hours, without exhibiting membrane toxicity, as demonstrated by a LDH release assay.

The transfection efficiency in non-phagocytic cells was elevated compared to naked DNA and all the other formulations. However, the mechanism of transfection studied by confocal microscopy has to be further investigated. The

RG 502H+PEI 10% microparticle formulation was used for in vivo immunization in mice. These experiments demonstrated that adsorption of DNA on the surface of cationic microparticles could reduce the amount needed for an immune response by DNA immunization. Still, the in vivo effect of DNA adsorption onto the surface of these cationic microparticles could not be set into relation with the in vitro transfection efficiencies observed earlier.

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CHAPTER 5

DESIGN OF AMINE-MODIFIED GRAFT POLYESTERS FOR THE EFFECTIVE GENE DELIVERY USING DNA-LOADED NANOPARTICLES

SUMMARY

DNA loaded nanoparticles were prepared using a newly designed platform of polymers with the aim to create an effective particulate gene delivery system.

The polymers were synthesized by carbonyldiimidazole (CDI) mediated coupling of diamines, diethylaminopropylamine (DEAPA), dimethlyamino-propylamine (DMAPA) or diethylaminoethylamine (DEAEA) to poly (vinyl-alcohol) (PVA) with subsequent grafting of D,L-lactide and glycolide (PLGA) (50:50) in the stoichiometric ratios of 1:10 and 1:20 (free hydroxyl groups / monomer units). The polymers were characterized by ^{1}H-NMR, GPC-MALLS (gel permeation chromatography - multiple-angle-laser-light-scattering), and DSC (differential scanning calorimetry). DNA loaded nanoparticles, prepared by a specifically modified solvent displacement method, were characterized with regard to their zeta (ζ) -potential and size. The transfection efficiency was assessed with plasmid DNA, pCMV-luc, in L929 mouse fibroblasts.

The polymers were composed of highly branched, biodegradable cationic polyesters exhibiting amphiphilic properties. The amine modification further enhanced the rapid polymer degradation and was held responsible for the interaction with DNA during particle preparation. The nanoparticles exhibited positive ζ-potentials up to + 42 mV and high transfection efficiencies, comparable to polyethlyenimine (PEI) 25kDa/DNA complexes at a nitrogen to phosphate ratio of 5.

The polymers combined amine-functions and short PLGA side chains resulting in water insoluble polymers, capable of forming biodegradable DNA nanoparticles through coulombic interactions and polyester precipitation in aqueous medium. The high transfection efficiency was based on fast polymer degradation and the conservation of DNA bioactivity.

INTRODUCTION

DNA vaccines have been subject to intensive research efforts recently and it has become increasingly clear that adjuvants are necessary to reduce the DNA dose, while reaching protective immune responses [1]. Adjuvants, such as micro – and nanoparticles have been studied intensively as DNA delivery systems providing i) a sustained and predictable DNA release; ii) targeting antigen of presenting cells using particles < 10 µm and iii) stabilization of DNA in physiological environment [2]. Several encapsulation techniques, mainly using biodegradable PLGA, have been reported, such as spray-drying [3] and modified double emulsion methods [4], all of which utilized high-speed homogenization or sonication. These shear forces were found to compromise plasmid integrity and bioactivity [5,6]. Additionally, DNA was damaged in the acidic environment created by PLGA degradation products [3].

Here, we describe a gentle solvent displacement method for the encapsulation of DNA relying on a new class of biodegradable polymers with rapid degradation properties [7]. This method allows the encapsulation of DNA without high speed / shear homogenization using amine-modified branched polyesters. These polymers interact with DNA by electrostatic interactions and facilitate nanoparticle formation due to their amphiphilic character. We systematically investigated these polymers to characterize the influence of polymer structure on functional properties such as nanoparticle size and charge, as well as the protection of plasmid DNA by the measurement transfection efficiency in vitro.

MATERIALS AND METHODS

Polymer Synthesis and Characterization

Biodegradable comb-branched polymers consisting of amine-modified poly (vinyl alcohol) (PVA) backbones, grafted with PLGA side chains in a ratio [n(OH)/n(monomer)] of 1:10 and 1:20 were synthesized and characterized as previously described [7]. The amine modifications consisted either of 3-diethylamino-1-propylamine (DEAPA = P), 2-diethylamino-1-ethylamine (DEAEA = E) or 3-dimethylamino-1-propylamine (DMAPA = M). Briefly, after activation of the diamine component using carbonyl diimidazole (CDI) in tetrahydrofuran (Fig. 1) the activated components were added to PVA, (Fluka, degree of polymerization: P=300) in N-methylpyrrolidone and reacted for 4 days at 80°C. Then lactide and glycolide (50:50) were grafted in stoichiometric ratios of 1:10 and 1:20 (free hydroxyl groups / monomer units) by bulk polymerization onto the amine-modified PVA-backbones at 150°C using tin(II) 2-ethylhexanoate as catalyst.

The source-based IUPAC nomenclature for e.g. DEAPA modified polymers is the following: Poly (vinyl 3-(diethylamino)propylcarbamate-co-vinyl acetate-co-vinyl alcohol)-graft-poly(DL-lactide-co-glycolide). As abbreviation we use A(x)-y. (A indicates the type of amine substitution (P=DEAPA, M=DMAPA, E=DEAEA), x is the number of monomers in the backbone carrying amine substitutions, y is the PLGA side chain length calculated from feed). Resomer®502H (RG 502H, Mw 15,200 g/mol, specifications supplied by the manufcturer) was purchased from Boehringer Ingelheim (Ingelheim, Germany).

^{1}H-NMR spectra were generated in d_6-DMSO with a Jeol Eclipse+500 NMR Spectrometer (JEOL, USA) at 50°C using 64 scans (500 MHz). GPC-MALLS was carried out with a combination of DAWN EOS, Optilab DSP (Wyatt Europe GmbH, Germany) and PSS SDV linear M column (PSS, Mainz,

Germany, flow rate 0.5 ml/min, solvent: dimethylacetamide +2,5 g/L LiBr at 60°C). DSC measurements were conducted with a Perkin-Elmer DSC 7 (USA). Polymer degradation was measured gravimetrically after incubation of polymer films in PBS-buffer at pH 7.4 (37°C) over 21days according to Wittmar et al. [8].

DNA Nanoparticle Preparation and Characterization

Nanoparticles were prepared by a modified solvent displacement method [9]. Briefly, 500 µl of an aqueous solution containing 0.5 µg/µl plasmid DNA was added to 2.5 ml of an acetone solution containing 50 mg of the water insoluble polymer. The product was injected into 10 ml stirred 0.1% Pluronic™ F68 (BASF, Germany) in distilled water. The resulting nanoparticle suspension was stirred 3 hours under constant laminar air flow to remove residual acetone. Particle size and ζ-potential measurements were carried out in a Malvern Zetasizer 4 (Malvern, Germany), according to Jung et al. [9] after calibration with a Malvern –50 mV transfer standard. Scanning electron microscopy (SEM) was performed with a CamScan 4 (Cambridge, UK) after gold sputter coating using a AUTO 306 (Edwards, UK). High resolution transmission electron microscopy imaging (TEM) was performed after cryo-sectioning of the nanoparticles with a JEM 3010 (Jeol, Japan) on a collodium grid.

In Vitro Transfection Efficiency

L929 mouse fibroblast (DSMZ, Germany) cells were plated at a cell density of 50 000 cells/ 2 ml in 12 well dishes 24 h prior to transfection. Aliquots of the particle suspension containing 4 µg pCMV-luc theoretical load were added to 0.5 ml glucose 5 % medium pH 7.4. The cells were pre-incubated with the nanoparticle suspension for 5 min, after which 1.5 ml cell culture medium containing 10% fetal calf serum (FCS) was added. The nanoparticle suspension

was dispersed in the glucose medium before the addition of the medium, since instabilities of nanoparticle suspension were observed in medium. The nanoparticle suspension was removed after 4 hours of incubation and replaced with fresh medium containing 10% FCS. Cells were harvested after 48 h and luciferase transfection efficiency was assessed according to Kunath et al. [10]. Results were presented as luciferase / protein ratio [ng/mg].

RESULTS AND DISCUSSION

In this study we present a new class of water-insoluble, amphiphilic polyesters, developed specifically for DNA encapsulation. We hypothesized that the loading efficiency of DNA nanoparticles could be greatly increased by three characteristics of the comb-branched polymers. Firstly, electrostatic interactions are thought to stabilize and protect DNA during the encapsulation process. Secondly, fast polymer degradation rate should allow the release of bioactive DNA and thirdly, tertiary amino-functions should facilitate gene delivery. We therefore developed polymers containing an amine-modified backbone for ionic interactions and possible buffering capacities in the endosomes and relatively short but multiple, biodegradable PLGA side chains for fast polymer degradation. The unique properties of these polymers were confirmed during the nanoparticle formation process. DNA was solubilized by the polymer in the acetone solution due to the amphiphilic characteristics in the acetone/water mixtures used for the solvent displacement method, suggesting a strong DNA/polymer interaction. The subsequent injection in aqueous medium resulted in nanoparticle formation. The biodegradable DNA nanoparticles exhibited effective gene delivery, demonstrated by high transfection efficiencies in-vitro.

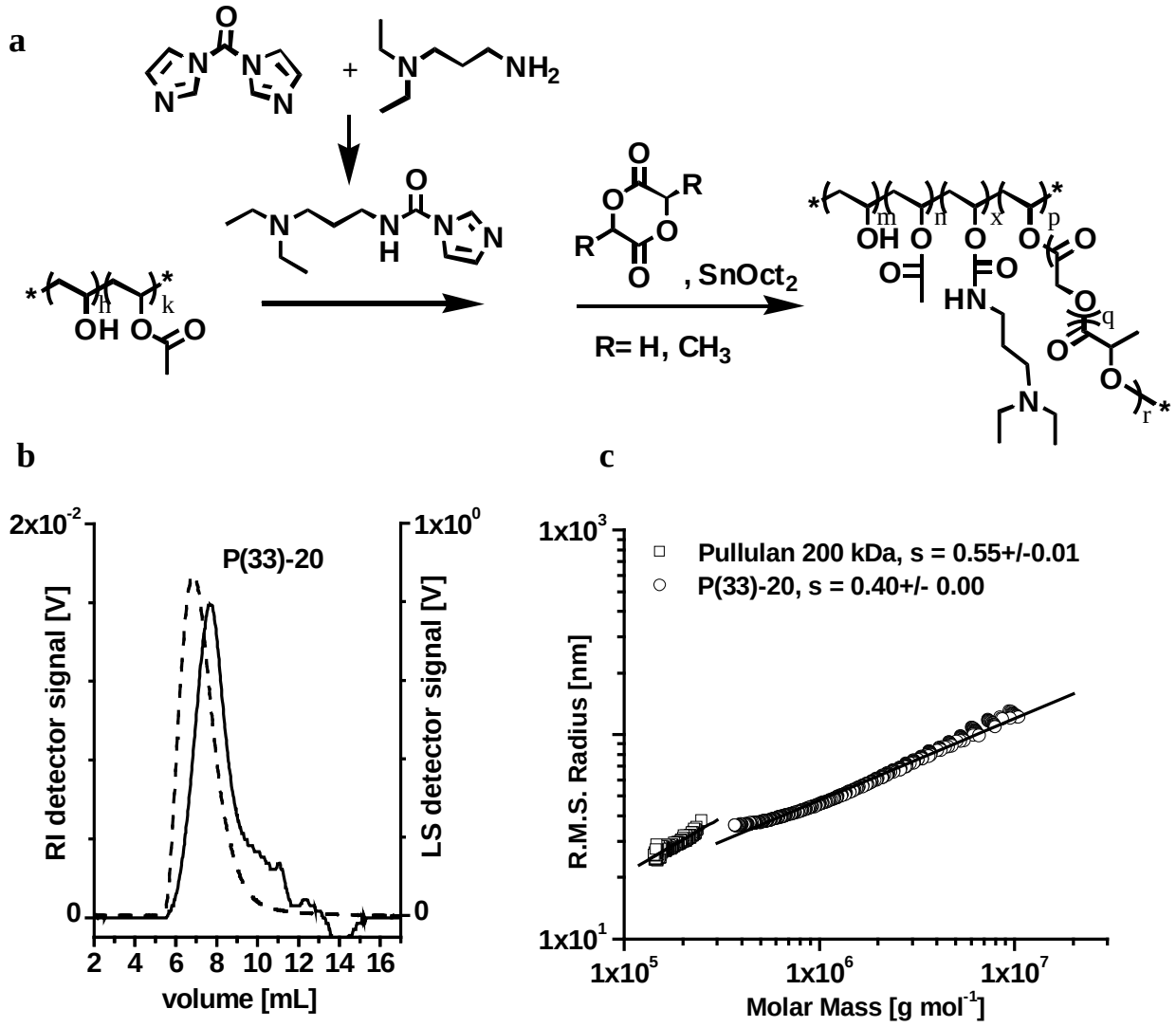

Fig.1: *The synthesis of poly (vinyl 3-(diethylamino)propylcarbamate-co-vinyl acetate-co-vinyl alcohol)-graft-poly(D,L-lactide-co-glycolide) using a three step process with PVA, amine and CDI as precursors b) GPC elution profile of P(33)-20 (signal of the refractive index detector: straight line, light scattering signal: dashed line) indicating a monomodal distribution of the molar mass of the polyesters, but also a small, low molecular weight part c) plot of the radius of gyration against MW. The reduced slope of the amine-modified polyester (P33)-20) demonstrated the highly branched structure in comparison to random coiled pullulan.*

We synthesized 24 cationic, as well as two neutral derivatives of amine-modified comb-branched polyesters, and characterized their functional properties in relationship to their structure (Fig.1). We grafted relatively short PLGA side chains consisting of approximately 10 or 20 repeating units on the amine-modified PVA-backbone. Consequently, already a small number of hydrolytic cleavage events would result in water soluble polymer fragments, thereby releasing the encapsulated DNA. The total number of biodegradable PLGA side chains grafted on an amine-modified PVA backbone ranged from 150 - 240, resulting in a cationic and water insoluble polyester. The general characteristics of the polymers properties with different amine substitutions (DEAPA / DMAPA / DEAEA) were similar. The DEAPA substituted polyesters were all soluble in acetone and thus, suitable for the nanoparticle preparation process. Therefore, we selected this type of polymers for further study.

The brush-like structure of the graft-polymers was verified using ^{1}H-NMR spectroscopy, as well as GPC-MALLS depicted in (Fig.1b/c) for P(33)-20. The degree of PLGA side chain substitution was calculated from the ^{1}H-NMR spectrum showing that only 5 to 35 % hydroxyl-groups of the PVA still remained free after reaction. The PLGA side chain lengths (SCL) were calculated from these data, demonstrating good correspondence with the theoretical values (Table 1). However, increasing amine substitution led to a decrease of SCL. A possible explanation could be an inhibitory effect of the amino-function on the tin catalyst which competed with lactide/glycolide monomers. The molecular weights of the polymers were calculated from a combination of this data, based on the known amine substitution of the PVA backbones. The values for molecular weight (MW) were confirmed by GPC-MALLS (Fig.1b). GPC measurements demonstrated the monomodal MW distribution of the polyesters. The molecular weights did not show an expected trend towards lower MW with increasing amine-substitution because of i) the fast degradation of the polyesters, ii) the resolution of GPC and iii) decreasing

acetate content with increasing amine substitution. The nanostructure of the polymers in solution was characterized by the evaluation of the radius of gyration in a double logarithmic scale plotted against the molar mass of the polyesters (Fig.1b). The resulting slope of the linear fit was compared to the slope of random coiled pullulan (0.55). The flatter slope exhibited by the amine-modified polyesters (P(33)-20: 0.40) indicated a compact, highly branched nanostructure of these polymers.

Polymer degradation at 37°C in PBS buffer at pH 7.4 was greatly accelerated as compared to common linear PLGA. NMR studies demonstrated the reduction of the SCL of P(12)-10 from originally 10.8 units to 8.6 units in seven days and to 5.4 units after another week. These measurements cannot be exclusively explained by physical erosion. Such an erosion would either not show deceased SCL or only a small SCL reduction. This behavior may substantially reduce the exposure time of the encapsulated substance to the detrimental effects of acidic degradation products generated by PLGA bulk erosion. The degradation behavior thus was remarkable, since the molecular weights of the graft-polyesters were approximately ten-fold higher than the linear PLGA (RG 502H) (Table 1). This property corresponded to our hypothesis of a substantial reduction in time for the drug release. An increase in the PLGA side chain length from approximately 10 to 20 repeating units increased the degradation time as expected. P(33)-20, for example, showed a degradation half-life of 13 days, compared to one day for the P(33)-10 analogue (Table 1). The degradation rates increased more than proportionally with increasing amine substitutions of the polymer. For example, the degree of amine substitution in P(33)-10 was three times greater than in P(12)-10, however, P(33)-10 exhibited a nine-fold increase in the rate of degradation. This effect could be explained by the rapid, initial PLGA mass loss of the P(x)-10 polymer in comparison to the slower mass loss of P(x)-20 polymers, attributed to a catalytic effect of the amino-functions, promoting the acidic ester degradation, caused by their protonation.

Polyester	T_g [°C][a]	MW [kg mol^{-1}]		SCL[d]	Degradation half-life[e,f] [d]	Nanoparticle size[f] [nm]	Zeta Potential[f] [mV]
		M_n[b]	M_n[c] M_w[c]				
P(6)-10	30.6	(107)	211 281	11.2	>21	n.d.	n.d.
P(12)-10	30.8	179	196 263	10.8	9	163 ± 1	22 ± 1
P(33)-10	27.7	179	195 367	9.4	1	152 ± 3	35 ± 3
P(68)-10	11.5	172	282 799	7.4	n.d.	309 ± 16	42 ± 2
P(12)-20	33.0	422	227 304	19.3	>21	n.d.	n.d.
P(33)-20	32.8	385	375 712	17.2	13	351 ± 7	31 ± 5
RG 502H[f]	36.5	6.1	6.6[f] 15[f]	84.6	19	602 ± 3	-55 ± 3

Table I: *Characterization of the amine-modified polyesters, demonstrating the low glass transition temperatures, extremely high molecular weights (MW) combined with fast polymer degradation at 37 °C in PBS buffer. DNA nanoparticles exhibited smaller sizes and high ζ (zeta)-potentials, compared to PLGA nanoparticles.*

[a] Glass transition temperature (heat rate: 10 °C/min, -10 to 200 °C, second run)

[b] MW calculated from the ^{1}H-NMR data

[c] MW from GPC-MALLS (DAWN EOS, Optilab DSP, column PSS SDV linearM, solvent DMAc+2.5 LiBr g/L, 60°C, 0.5 mL/min)

[d] PLGA side chain length calculated from ^{1}H NMR

[e] Days for 50% mass loss of a polymer film (extrapolated from plot, n=3)

[f] mean of three independent measurements ± standard deviation

[g] Commercial PLGA (1:1) lactic acid : glycolic acid subunits. MW: specifications supplied by the manufacturer (Boehringer Ingelheim)

This would lead to new carboxyl-functions restarting the catalytic cycle.

Further, the protonated amino-functions will promote water uptake into the polymer effecting an increased rate of hydrolysis. All polymers displayed glass transition temperatures near 30 °C, implying that they exist in the glassy state in physiological environment (Table 1). In general, polymers with longer PLGA side chains and reduced amine substituents had higher transition temperatures. Thus, the amine-groups were thought to have acted as a plasticizer in the polymer. The influence of the polymer chain motility has to be further investigated for possible interactions with cellular membranes and the influence on the gene delivery process.

An important feature of the polymer characteristics were the tertiary amine-modifications of the polymers, hypothesized to stabilize DNA within the polymer matrix and to facilitate the gene transfer. Ionic interactions with the polymer were presumably the reason for the solubilization of DNA in the acetone/water mixture. For example, DNA could be completely dissolved in an acetone/water 5:1 [$^{v}/_{v}$] solution of the polymer, whereas DNA alone precipitated. Therefore, no further homogenization process was necessary to disperse DNA before the subsequent coacervation of the water insoluble polymer in the 0.1% poloxamer solution. Nanoparticles were only obtained with polymers exhibiting amine substitutions of 4% (P(12)-10) and higher, underlining the importance of the amphiphilicity, induced by the amine substituents. The structure of the polymers was described to be brush-like, due to the short and numerous PLGA chains. Therefore, we did not expect a micellar assembly of the polymers neither in acetone, nor in the non-solvent water. In contrast, water soluble, poly(l-lysine)-g-PLGA polymers had a more distinct amphiphilic structure, containing a shorter hydrophilic backbone with few and long PLGA chains of approximately 210 monomers [11].

The nanoparticles exhibited hydrodynamic diameters ranging from 152.4 nm (P(12)-10) to 351.3 nm (P(33)-20), whereas PLGA (RG 502H) nanoparticles prepared by the same procedure were approximately 200 nm larger (Table 1).

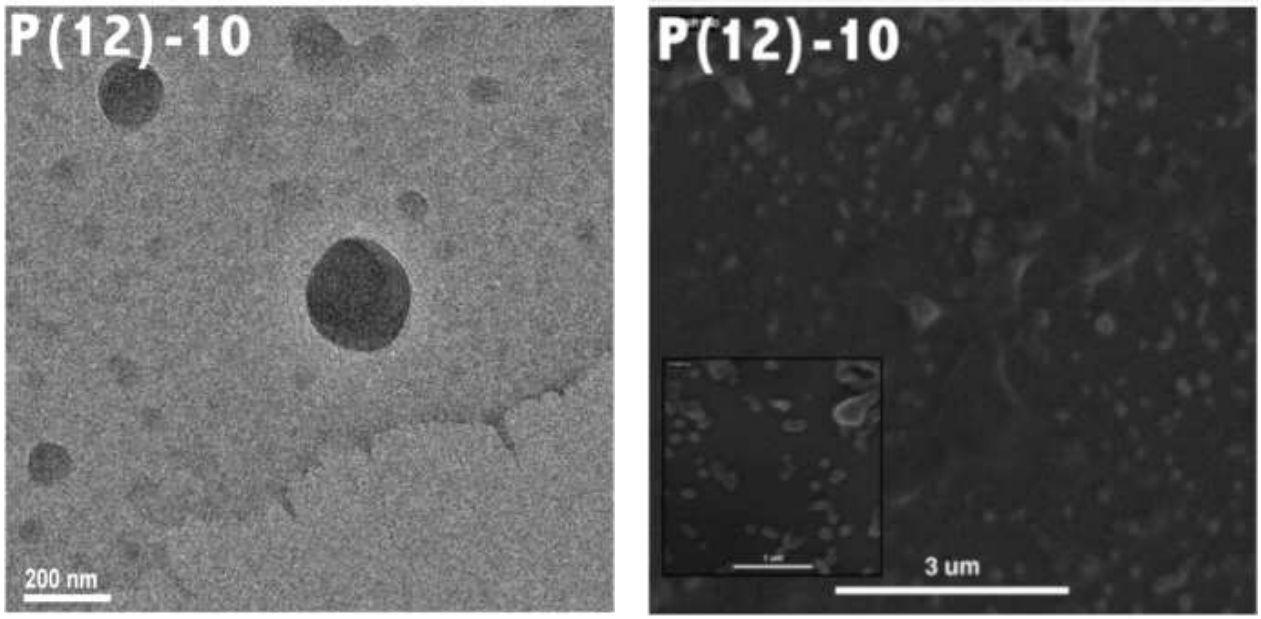

Fig.2: *TEM (left) and SEM (right, 1µm scale of the inlay) micrographs of DNA P(12)-10 nanoparticles confirmed the particulate structure of the nanoparticle and the size measured by photon correlation spectroscopy.*

Hence, despite a 33-fold higher molecular weight, the amphiphilic qualities of the polymers, influencing the interface tension, resulted in nanoparticles of reduced size.

ζ-potentials of all preparations were clearly positive, with the exception of the linear PLGA, arising from the DNA phosphate groups, which were inverted by the cationic polymers. Particle sizes measured by photon correlation spectroscopy were confirmed by scanning electron microscopy (SEM) and transmission electron microscopy of nanoparticle cryo-sections (TEM). The particle morphology was examined by these methods as well for the polymer P(12)-10 in Figure 2. Particles were uniform in size and had smooth surfaces.

All DNA nanocarriers were used in vitro for transfection assays, as efficient gene delivery remains a prerequisite for subsequent in vivo immunization. By directly using the nanoparticles in vitro, we could detect the gene transfer properties of the amine-modified polymers, as well as the DNA bioactivity after nanoparticle preparation. Free plasmid and DNA complexes with PEI 25kDa, a potent polymeric transfection agent, were used as references to compare the luciferase expression levels with other polymer types [12]. On account of this, we could consider the nanoparticles as a potent transfection agent. All DNA nanocarrier formulations resulted in increased transfection efficiencies compared to naked DNA (Fig.3). The efficiency increased exponentially with the amount of amine substitution of the polymer.

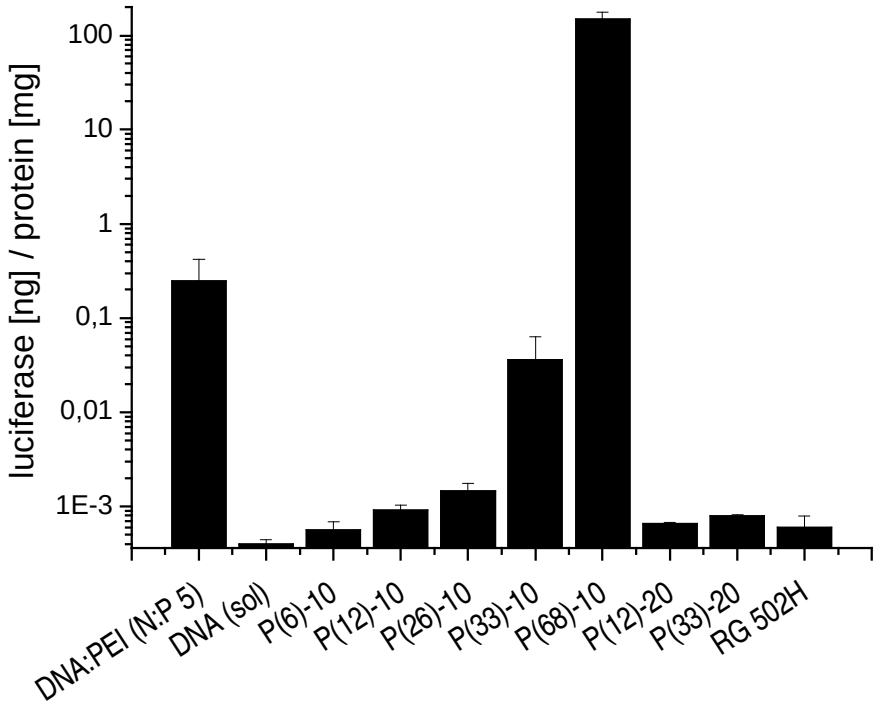

Fig.3: *Transfection efficiency of pCMV-luc DNA, encapsulated in amine-modified nanoparticles was greatly enhanced compared to free DNA, DNA/PEI 25kDa complexes (N/P 5) and a DNA RG 502H particle preparation.*

The 500,000-fold increase in transfection efficiency of the P(68)-10 plasmid nanoparticles, compared to free DNA, was remarkable, especially when considering the fact that the amount of polymer in relation to DNA was reduced by the factor 0.4 to avoid nocuous effects of an excess of cationic charges. Nanoparticles of P(x)-10 polymers clearly displayed higher efficiencies than their P(x)-20 analogous.

The careful elucidation of the transfection mechanism of the polymers is yet to be investigated, however, we assume that these findings do not depend on increased ζ-potentials or particle size effects. They but must be dependent on the particle structure and DNA polymer interactions as well. The polymers consisted of dimethlyaminopropylamine substituents, representing tertiary amines. These have been shown to be essential for the endosomal escape of polyplexes by the osmotic rupture, the `proton sponge´ effect [13]. This effect could be intensified by the fast polymer degradation resulting in an increase of the osmotic pressure in the endosome, as proposed by Koping-Hoggard [14]. However, other mechanisms of endosomal release have eventually to be considered, for example, fusogenic activities, taking into account the low glass transition temperatures and hydrophobic moieties of the polymer [15], or the `hydrogel effect´ of swelling polymer in the endosome [16]. Therefore, we concluded that the combination of different properties within one biodegradable polymer, resulting in a fast degradation, ionic interactions with DNA and the formation of water insoluble nanoparticles, provided considerable advantages with regard to the transfection efficiency *in vitro*. Further experiments investigating the transfection efficiency under *in vivo* conditions and the encapsulation with other compounds, such as peptides, susceptible to acid degradation are in progress.

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CHAPTER 6

CHARACTERIZATION OF DNA ENCAPSULATION INTO AMINE-MODIFIED POLYMER NANOPARTICLES USING THE SOLVENT DISPLACEMENT METHOD

SUMMARY

To circumvent DNA degradation during particle formation, the DNA was adsorbed onto pre-formed particles [1,2]. However, the adsorption via electrostatic interactions of DNA or other macromolecules onto colloidal structures can cause instabilities, such as flocculation, by either charge neutralization or bridging [3]. In this study, we intended to characterize a new approach for DNA nanoparticle formation. This new process allowed the encapsulation of DNA without using high-speed shear forces. Thereby, we aimed to reduce both particle flocculation and DNA degradation by DNA encapsulation.

One representative polymer, P(26)-10, of a new class of amine-modified polyesters was used for the study of nanoparticle formation. The formulation method used in this study had previously only been described for the efficient encapsulation of hydrophobic compounds. We investigated the influence of several process parameters on the nanoparticle size. The DNA nanoparticle size was dependent on the volume of the organic solvent, as well as on the volume of the aqueous solutions. The viscosity of the organic solvent further influenced the particle size and the encapsulation efficiency. This system exhibited some variations, when compared to the standard solvent displacement techniques. These were explained by polyelectrolyte interactions of the polymer with DNA in the acetone/water medium. The ratio of the water/acetone medium apparently influenced the polyelectrolyte interactions of the DNA with the cationic polymer. The subsequent particle formation was dependent on the polymer coalescence, which we hypothesized to be influenced by the polymer / DNA interactions and solvent composition. The new class of amine-modified polyesters used in this study, was shown to be a promising tool for a one step DNA encapsulation into nanoparticles without using shear forces.

INTRODUCTION

Recently, DNA has been successfully adsorbed on cationic microparticles and nanoparticles for the use as adjuvant systems for DNA immunization and gene delivery [1,4,5]. Thereby, DNA interacts via electrostatic forces with particle surfaces. The DNA / particle ratio was determined by the particle's size and the particle's surface charge. However, we and others have found that these colloidal systems exhibited instabilities, namely flocculation, when used for the adsorption of macromolecules, such as DNA [6]. The two mechanisms mainly involved in this phenomenon were charge neutralization and polymer bridging [3]. To avoid thee drawbacks we investigated methods to encapsulate DNA into nanoparticles.

The solvent displacement method has typically not been an efficient technique for the direct encapsulation of water soluble drugs [7]. The method is based on polymer deposition on interfaces, due to the aggregation of polymers by organic solvent displacement. The driving force of the particle formation is the mass transport of an organic solvent into a second solvent, mostly water. This phenomenon was first described by Marangoni in 1871 [8]. The mass transport into the larger second phase induced interphase turbulences that resulted in interphase disruption and small solvent droplet formation. Commonly used organic solvents are water soluble and non-chlorated which exhibit low boiling points for efficient extraction and evaporation of the organic solvent form the preparation.

In this study, we used P(26)-10, as a representative polymer for the new class of amine-modified polyesters [9]. These polymers were characterized by amphiphilic and cationic structures, which enabled us to modify the solvent displacement method for the encapsulation of DNA [10]. All nanoparticles were formulated according to a standard method, changing one parameter at a time.

MATERIALS AND METHODS

Polymer and DNA

The biodegradable polyester P(26)-10, was composed of an amine-modified poly(vinyl alcohol) (PVA) backbone with grafted poly(lactic-co-glycolic acid (PLGA) side chains, synthesized according to Wittmar et al. [9]. The cationic polymer backbone was composed of 26 units 3-diethylamino-1-propylamine (DEAPA = P) substituted on poly (vinyl alcohol) (PVA, Fluka, degree of polymerization: P=300). The grafted side chains consisted of D,L-lactide and glycolide (50:50) in a ratio of 1:10 (free hydroxyl groups / monomer units), resulting in approximately 200 PLGA side chains per hydrophilic backbone.

Herring testes (HT) DNA (Sigma-Aldrich, Taufkirchen, Germany) was used for the mechanistic and feasibility studies. It was dissolved in low ionic strength (I = 0.01) TE-buffer pH 7.4 (3.3 mM Tris(hydroxymethyl) aminomethane (Tris-HCl), 0.3 mM disodium edetate (Na_2EDTA) pH 8).

Nanoparticle Preparation

DNA nanoparticles were prepared using a modified solvent displacement technique. Therefore, 50 mg of the polymer were dissolved in 1.25 ml acetone. The HT DNA was diluted to a final concentration of 2 µg/µl in 250 µl of low ionic strength buffer at pH 7.4 and dispersed into the acetone solution containing the amine-modified polymer. The dispersion was prepared by fast injection of the aqueous solution into the polymer solution while mixing several times with the pipette. The resulting dispersion was then slowly injected into 20 ml of a magnetically stirred 0.1 % aqueous Pluronic™ F68 (BASF, Germany) solution, using a 14-gauge needle. Under these conditions spontaneous nanoparticle formation could be observed. The nanoparticle suspension was stirred for 3 hours under constant air flow for complete removal of residual acetone. Particles were characterized directly after the preparation.

Particle Size Measurement

The effective hydrodynamic diameter was measured by photon correlation spectroscopy (PCS), using a Malvern Zetasizer 4 (Malvern Instruments, Germany) at 25°C, equipped with a 5 mW helium neon laser and the Malvern software. Samples were measured in an AZ 110 cell at 633 nm and a scattering angle of 90°. The samples were diluted in ultrapure water if needed, to measure within a defined count rate interval of 100 – 400 kilo counts / second and to avoid multiscattering. The viscosity (0.88 mPa s) and the refractive index of ultrapure water (1.33) were used for data analysis. The PCS V. 1.26 - software was used to calculate the particle mean diameter and width of the fitted gaussian distribution. All measurements were carried out in triplicate.

Scanning electron microscopy (SEM) was performed with a CamScan 4 (Cambridge, UK) after gold sputter coating using a AUTO 306 (Edwards, UK) for particle size confirmation.

DNA Encapsulation Efficiency

The DNA distribution in either the nanoparticles or in the supernatant was evaluated using 1% agarose gel electrophoresis with ethidium bromide staining. The nanoparticle samples were centrifuged at 14,000 rpm (16,025 g) for 20 min in an Eppendorf 5415C centrifuge (Wesseling, Germany), to separate the particle pellet from the supernatant. The supernatants were directly applied into the gel adding 5 µl glycerol and 10 µl of dextran sulfate (Mw 5,000, Sigma-Aldrich, Taufkirchen, Germany) 0.25 mg/ml to 20 µl of the probe. DNA encapsulated and associated with the polymer nanoparticles was extracted from the polymer using Roti-phenol® (Roth, Karlsruhe, Germany). The pellet was firstly incubated for one hour with 80 µl of a 0.25 mg/ml dextran sulfate solution in isotonic TE buffer (1mM Na_2EDTA; 10mM Tris; 143mM sodium chloride). Roti-phenol®, 100 µl, was added thereafter and incubated for one additional hour under constant stirring of 10 rpm in a Rotatherm® (Liebisch, Bielefeld,

Germany) at room temperature. The two phases were separated by centrifugation at 14,000 rpm for 20 min in an Eppendorf 5415C centrifuge. Glycerol, 5 µl, was added to 20 µl of the aqueous phase and applied into the 1% agarose gel. Electrophoresis (Blue Marine 200, Serva, Germany) was carried out at 100 V for one hour in 0.1M TE-buffer. 8 µl of a 1 % (w/v) ethidium bromide solution were included into all gels to visualize the DNA localization by photography with UV transillumination.

RESULTS AND DISCUSSION

The solvent displacement method represents a very promising tool for the encapsulation of drugs, susceptible to shear and heat degradation. For the hydrophilic compound, such as DNA, this process has been shown to be non-effective [7]. We hypothesized, that the complexation of DNA with polycations would reduce its hydrophilicity. Alternatively, a polycation / DNA complex could represent a surface for polymer deposition and aggregation. Thus, we were primarily interested in the encapsulation of complexed DNA within the newly synthesized fast degrading polyesters. Interestingly, we observed that neither the aqueous DNA solution nor the polymer precipitated when the two solutions were mixed. This was noteworthy, as DNA is insoluble in acetone and the polymer is insoluble in water (Fig.1). This phenomenon was explained by polyelectrolyte interactions in the acetone/water medium. Polyelectrolyte complexes in low-polar solvents, such as chloroform have been studied by Sergeyev et al. [11]. The interaction and solubilization of polyelectrolyte / surfactant complexes was ascribed to the high stability of salt bonds in the low polar environment. Further, the complexation capacity was hypothesized to arise from the DNA inherent property to reside in a condensed state [11]. As in this in the present study, DNA was insoluble in the organic solvent. Still, the solvent

acetone is much more polar (dielectrical constant: 20.7) compared to chloroform (dielectrical constant: 4.81) which was used by Sergeyev.

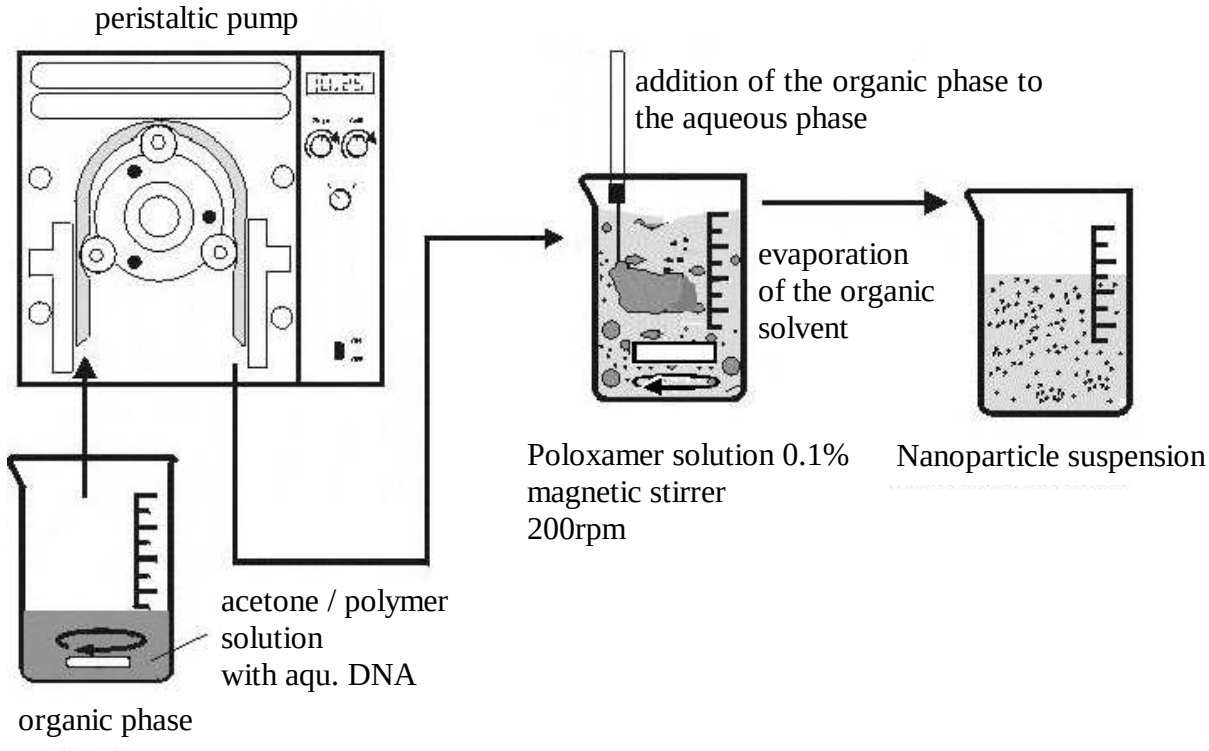

Fig.1: *Scheme of the nanoparticle formation process by solvent displacement. The mixture of the DNA and the polymer in a water / acetone medium is injected into a stirred stabilizer solution in which nanoparticles form spontaneously.*

Desbrieres et al. have studied pre-formed and lyophilized polyelectrolyte complexes in more polar solvent, such as dimethylformamide (dielectrical constant: 37). In this solvent both components were soluble and thus behaved similar to polyelectrolyte complexes in water [12]. Our system was considered to be similar to dimethylformamide, as we combined acetone and water. The particularity of the present method, compared to others, arises from the insolubility of DNA in the acetone/water mixture. This was demonstrated by DNA precipitation in the absence of the polymer in the acetone/water mixture. In both studies previously discussed, either both components were soluble in

water, or both were soluble in the organic solvent. P(26)-10 was insoluble in water. This enabled us to further use the solvent displacement method for nanoparticle formation by polymer coalescence in aqueous medium.

Investigators have studied the influence of formulation parameters of the solvent displacement technique on nanoparticle formation. In this study we varied several parameters to characterizes our system, especially the nanoparticle size [13-17].

All nanoparticles were formulated according to the standard method described, changing one parameter at a time. Nanoparticles prepared with the standard method had hydrodynamic diameters of approximately 160 nm (Fig.2). The standard volume of 250 µl of low ionic strength TE buffer corresponded to 20% of the 1.25 ml volume of acetone. When the acetone volume was doubled to 2.5 ml, the size of the particles was decreased to 135 nm. A further increase of the acetone volume led to larger particles again. It is known from the literature that lower solvent viscosities, as well as higher volumes of organic solvents result in a better disruption of polymer droplets and inconsequence, in smaller nanoparticles [13,14].

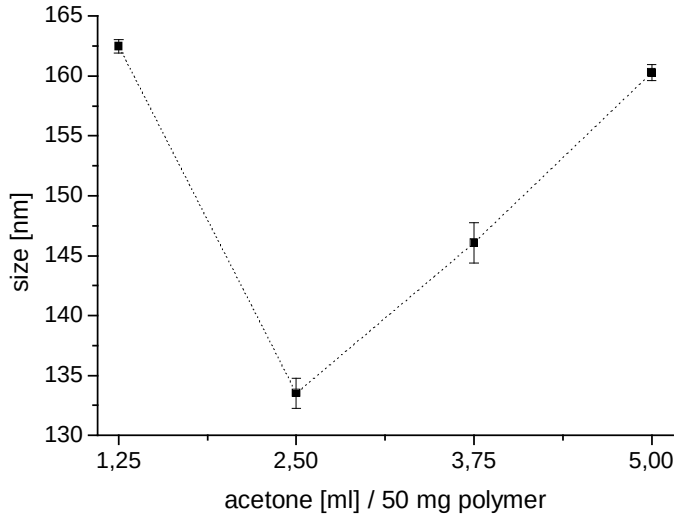

Fig.2: *Nanoparticle sizes using increasing amounts of acetone. An increase first led to a minimum particle size, further increase produced lager particles again.*

For the P(26)-10 system, this could not be observed for all of the samples. This effect was attributed to the influence of the polymer/DNA interaction. We assume that the coalescence rate of the polymer is also dependent on the size of the polyelectrolyte complex in the acetone/water medium. As ionic interactions increase with the hydrophilicity of the solvent, the complex size decreased [12]. Higher amounts of acetone could have resulted in larger complexes, arising from less intensive interactions of the polymers resulting in higher viscosities and reduced droplet disruption.

This hypothesis is in line with the nanoparticle sizes obtained by the encapsulation of polyethylenimine (PEI) 25 kDa (BASF, Ludwigshafen, Germany) complexes with DNA (Fig.3).

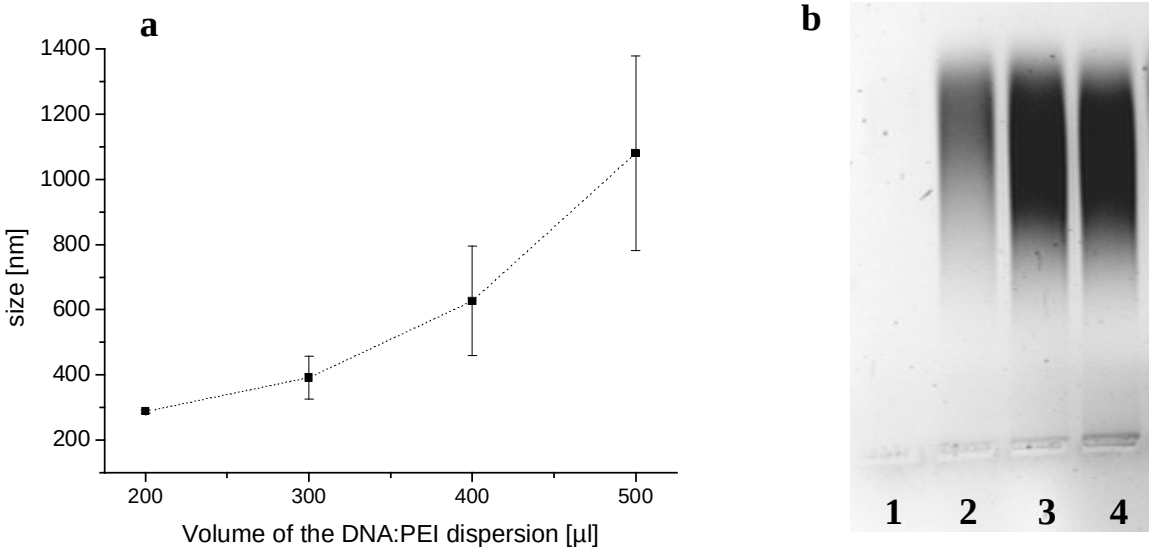

Fig.3: *(a) Size of nanoparticles prepared with equal amounts of PEI/DNA complexes dispersed in increasing volumes of low ionic-strength TE-buffer. The particle size increased with the volume of the aqueous phase mixed to the acetone solution. (b) DNA recovered from the nanoparticles prepared with 200 µl, 300 µl, 400µl and 500 µl of DNA complex solution, respectively bands 1 to 4. A volume of 500 µl aqueous solution led to the highest DNA encapsulation into the nanoparticles, as compared to formulations with lower buffer contents.*

All complexes were prepared at a nitrogen to phosphate ratio of 5 in ultrapure water. The complexes were diluted with TE buffer to the final volume of the aqueous phase. PEI 25 kDa is a well-known and very effective complexing agent for DNA. Therefore, the DNA / polymer interaction will have a less relevant impact on the nanoparticle formation process. Nanoparticle sizes increased with higher volumes of the aqueous complex solution. The diffusion rate of the solvent is dependent on the concentration gradient of the solvent. Thus, a dilution of acetone caused a slower diffusion, less droplet disruption and faster coalescence.

HT DNA of these nanoparticles was extracted and analysed by agarose gel electrophoresis (Fig.3b). The DNA encapsulation efficiency was the highest for 500 µl TE buffer preparation and decreased with lower aqueous volumes. This suggested that the coalescence rate of the polymer is higher, when increasing amounts of aqueous solutions are mixed to the acetone polymer solution. This restricted the distribution of the PEI/DNA complex into the aqueous phase during encapsulation.

A further well-known parameter influencing the nanoparticles size is the volume of the aqueous stabilizer solution. The concentration gradient of the acetone is greater when large aqueous phases are used. Hence, the mass transport is faster, resulting in more extensive droplet disruptions [13]. In our study this effect was observed for the preparations in 5 ml to 10 ml volumes of poloxamer solution. However, a volume of 20 ml resulted in larger nanoparticles again. Sterling et al. selectively described the two factors influencing the interfacial turbulences, i) the magnitude of surface viscosity and ii) the steepness of the concentration profile near the interface [13]. Therefore, we could only explain this finding by a hindrance of acetone diffusion into the aqueous phase, due to reduced mixing in the higher volume of aqueous solvent level during the preparation.

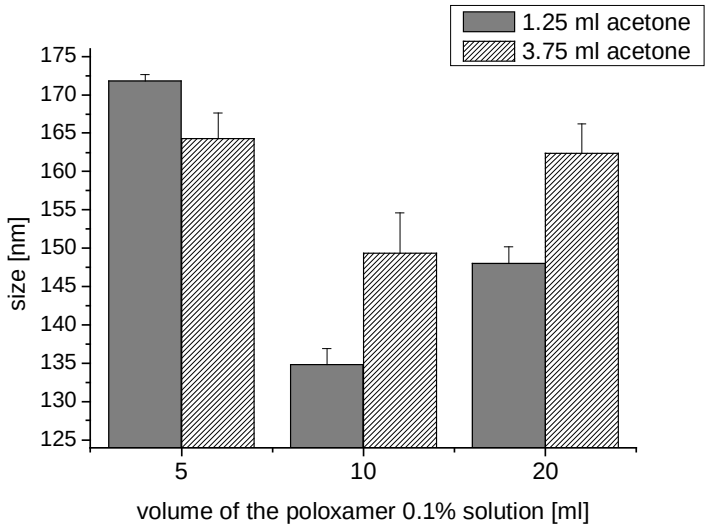

Fig.4: *Size of nanoparticles prepared in 5, 10, 20 ml poloxamer solution using either 1.25 ml acetone or 3.75 ml acetone.*

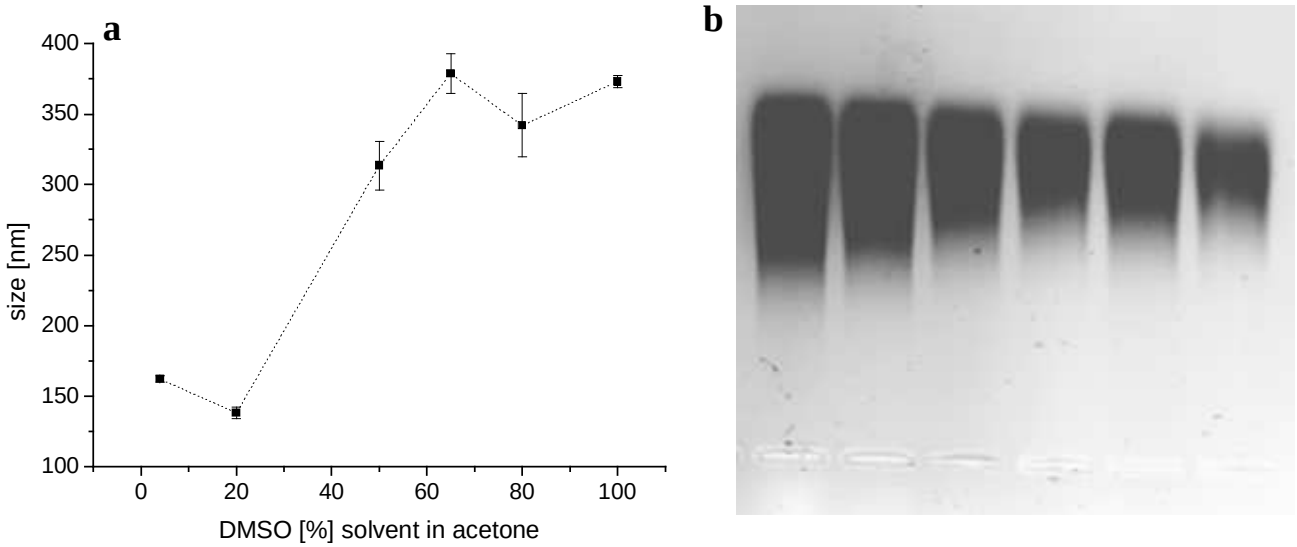

Fig.5: *Size of nanoparticles prepared using different DMSO / acetone ratios as organic polymer solvent. (a) The nanoparticle size increased with rising amounts of DMSO. (b) The amount of encapsulated DNA recovered from nanoparticles decreased with raising amounts of DMSO solvent.*

Increasing the amount of a model solvent DMSO in the acetone phase, led to larger hydrodynamic diameters of the nanoparticles (Fig.5a). This effect was attributed to the slower diffusion of the solvent mixture into the stabilizer solution because of a higher viscosity of DMSO (2.4 mPa*s) compared to acetone (0.34 mPa*s).

Particles in a size range of 350 nm could be prepared exclusively using DMSO as organic solvent. DMSO however, does not represent a realistic candidate for nanoparticle preparation, as it cannot be evaporated easily form the formulation. DNA of the nanoparticles was extracted and analyzed by agarose gel electrophoresis. The encapsulation efficiency of DNA decreased significantly when the amount of DMSO was raised (Fig.5b). This was ascribed to the prolonged coalescence rate of the polymer, due to slower mass transport of the solvent from the organic phase. This resulted in a greater diffusion of DNA into the supernatant.

In a further study an organic solvent, ethanol, was used as external phase instead of the 0.1% Pluronic solution (Fig.6). All other parameters for the nanoparticle preparation were held constant. We hypothesized that complete association of DNA with the nanoparticles was possible, as both, DNA and the polymer were not soluble in ethanol. The sizes of DNA loaded nanoparticles prepared without additives exhibited hydrodynamic diameters of approximately 600 nm. Interestingly, pre-complexed DNA with either PEI 25 kDa or the hydrophilic amine-modified PVA backbone resulted in smaller particles. We hypothesized that ethanol is a suitable external phase as it can be readily evaporated. Thus, concentrating the samples in smaller volumes or the transfer of the formulation into another medium would be easier to achieve. Still, aqueous systems have great advantages, especially with regard to the application on cells or in physiological environment.

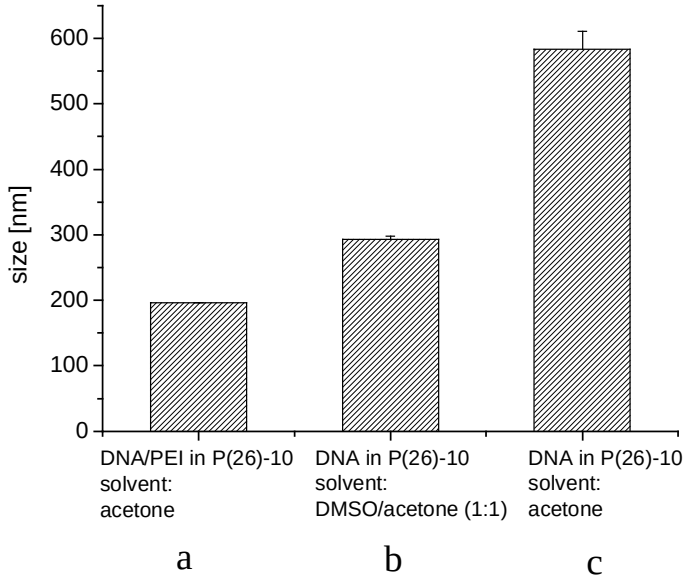

Fig.6: Nanoparticle suspensions prepared with ethanol used as external solution. (a) PEI/DNA complexes encapsulated in P(26)-10 dissolved in acetone.(b) DNA encapsulated in P(26)-10 dissolved in DMSO/acetone (1:1)[$^{v}/_{v}$], (c) DNA encapsulated in P(26)-10 dissolved in acetone.

The morphology of nanoparticles formulated by the standard setting was analyzed by SEM (Fig.7).

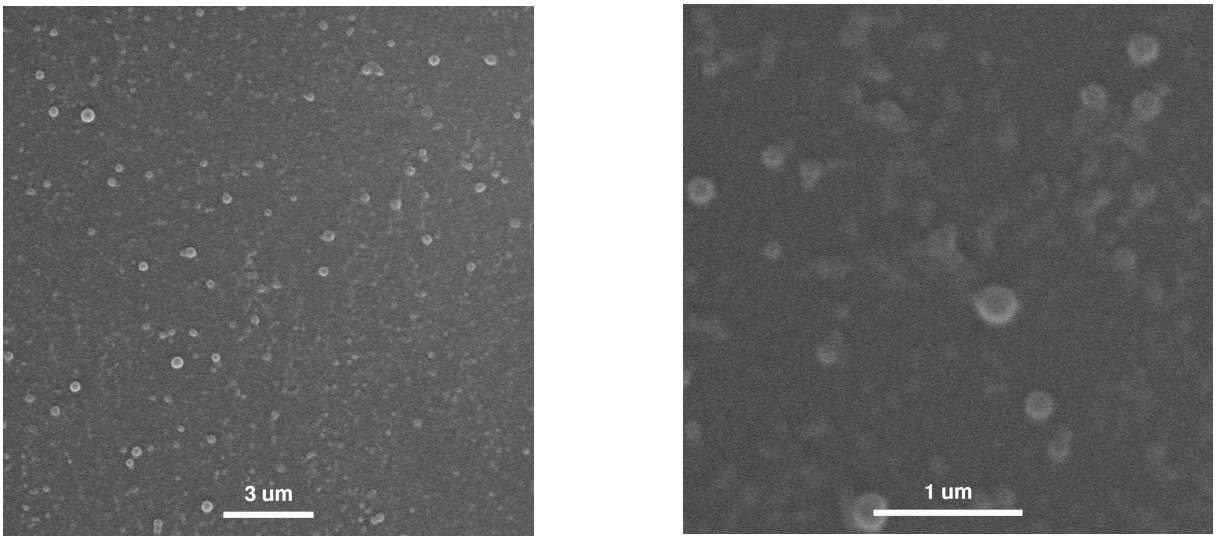

Fig.7: *SEM micrographs of nanoparticles prepared with the solvent displacement method using P(26)-10 as representative polymer for the amine-modified polyesters.*

These micrographs exhibited some well-defined structures, however, a majority of the particles appeared as a collapsed structures. This effect can be attributed to the very low glass transition temperature of the polymers [9]. This could result in the collapsed structure of the nanoparticles, upon exposure of the polymers to higher temperatures and energy during the gold sputter coating. Nanoparticle sizes however could be confirmed.

CONCLUSION

In this study we investigated the particle formation process for direct encapsulation of DNA into a representative polymer of a new class of amine-modified polyesters. We successfully encapsulated DNA using the solvent displacement technique which has been previously only described for the efficient encapsulation of hydrophobic compounds. We investigated the influence of several parameters on the particle size. Our findings were mostly in line with the literature. Still, this system exhibited specific properties, which could be explained by the polyelectrolyte interactions of the polymer with DNA. The characterization of DNA extracted from the nanoparticles represented some difficulties, as the P(26)-10 polymer precipitated in the aqueous solution. However, this study demonstrated that the polymer used is a very promising candidate for DNA encapsulation without the application of major shear forces.

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CHAPTER 7

DNA NANOCARRIERS FROM BIODEGRADABLE BRANCHED POLYESTERS FORMED BY A MODIFIED SOLVENT DISPLACEMENT METHOD

SUMMARY

The encapsulation of plasmid DNA into biodegradable micro- and nanoparticles has recently been a challenge for many groups, aiming to use the system for DNA vaccination and gene delivery. In this study we present a technique for DNA encapsulation into nanoparticles avoiding shear or ultrasonic forces by the use of biodegradable amine-modified polyesters. These biodegradable polymers combined specific characteristics, as ionic interactions with DNA and protonable amino-functions, providing an efficient nanoparticular system for gene transfer. The resulting DNA nanoparticles had hydrodynamic diameters ranging from 175 nm to 285 nm and highly positive ξ-potentials, depending on the nitrogen to phosphate (N/P) ratio used for the particle formation. Atomic force microscopy (AFM) confirmed particle sizes and showed well-defined shapes to more collapsed particle morphologies. DNA stability was investigated upon DNA release in PBS buffer and enzymatic degradation was assayed by agarose gel electrophoresis. This demonstrated that DNA was released in its supercoiled form and that it was protected from enzyme degradation. DNA nanoparticle cellular uptake was measured by flow cytometry using different N/P ratios. The efficient particle endocytosis was further followed over time by confocal microscopy.

The efficiency of the DNA nanoparticles was demonstrated by in vitro transfection assays in four cell lines. The gene delivery efficiencies of the amine-modified polymers were increased compared to free DNA. To demonstrate the power of the nanocarrier system, we compare the luciferase expression of the pCMV-Luc plasmid with PEI 25 kDa / DNA complexes used at equal N/P ratios. Thereby, we could show that one of the polyesters, P(68)-10, had higher efficiencies than the PEI 25 kDa complex.

INTRODUCTION

DNA vaccines have been under intensive investigation for over a decade [1,2]. During this time, it has become clear that a sufficient immune response with DNA vaccines can only be achieved with adjuvant systems. While, the injection of naked plasmid DNA in mice has been shown to induce an immune response, including the generation of antibodies and cytotoxic T lymphocytes [3,4], relatively high doses of DNA were needed to reach protective levels [5,6]. By the use of adjuvants, however, one could reduce the required DNA dose to practical levels. Several groups have successfully developed DNA adjuvant delivery systems based on micro – and nanoparticles [7]. Ideally, such systems should i) protect DNA from enzymatic degradation; ii) allow a sustained and predictable DNA release from the carrier and iii) target the delivery system to antigen presenting cells by appropriate particle sizes. The most commonly used biodegradable polymer for DNA encapsulation has been poly(lactic-co-glycolic acid) (PLGA). Many techniques have been used to encapsulate DNA within PLGA including spray-drying [8] and modified double emulsion methods [9], all of which rely on high-speed homogenization or sonication for the formation of particles suitable for phagocytic uptake. The effect of high shear forces on DNA, however, has been found to be very detrimental for plasmid integrity [10,11]. To circumvent DNA damage during this procedure, several methods have been proposed, such as complexation of DNA with cationic polymers prior to encapsulation [12], cryopreparation and the addition of ionic excipients [8]. All methods were able to diminish the effects of mechanical stress on DNA during encapsulation. However, upon release from these particles, DNA had been exposed to the PLGA acidic degradation products, namely lactic and glycolic acid, resulting in acid-catalyzed depurination and chain breaks [10]. Even the encapsulation of DNA using the least hydrophobic, uncapped PLGA

with a relatively small molecular weight exhibited DNA nicking shortly after its burst release [8].

To overcome these draw-backs, we developed a modified solvent displacement method for DNA encapsulation using a new class of biodegradable polymers which exhibit rapid degradation [13]. This method enabled us to encapsulate DNA without the use of high-speed homogenization. In the present study, we systematically investigated series of polymers belonging to branched polyesters with regard to their abilities to encapsulate, protect and deliver plasmid DNA.

MATERIALS AND METHODS

Polymers and DNA

Biodegradable comb polymers consisting of amine-modified poly(vinyl alcohol) (PVA) backbones grafted with PLGA at a backbone to PLGA side chain ratio [m/m] of 1:10 and 1:20 were used in this study [13]. The amine modification of the polymer backbone using either 3-diethylamino-1-propylamine (DEAPA = P), 2-diethylamino-1-ethylamine (DEAEA = E) or 3-dimethylamino-1-propylamine (DMAPA = M) was accomplished by N,N'-Carbonyldiimidazole coupling chemistry. PLGA grafting was performed using a ring opening polymerization procedure. The degree of amine-substitution was indicated by the number following the type of amine modification, representing the number of monomers of the PVA backbone carrying an amine-substitution. The PLGA chain length was described by the number after the degree of amine substitution, calculated from feeding. The commercially available PLGA (50:50), Resomer® 502H, (M_w 15,200, specifications supplied by the manufacturer) was purchased from Boehringer Ingelheim (Ingelheim, Germany).

Plasmid DNA, pLuc-CMV, a luciferase encoding plasmid, preceded by a nuclear location signal under the control of a CMV promoter, was kindly

provided by Chiron (Emeryville, Ca) and amplified by PlasmidFactory, (Bielefeld, Germany). The pLuc-CMV used in this study originated from a single endotoxin-free batch in TE-buffer pH 8 and was stored at -80°C until use. Pluronic™ F68 was purchased from BASF (Parsippany, NJ). All other chemicals were purchased by Sigma and were of analytical quality.

Nanoparticle Preparation

Nanoparticles of amine-modified PVA-graft-polyesters and PLGA were prepared by a modified solvent displacement method [14]. Briefly, herring testes (HT) DNA or plasmid DNA, was diluted to a final concentration of 0.5 µg/µl in 500 µl distilled water and dispersed into 2.5 ml acetone solution containing the amine-modified polymer. The dispersion was prepared by simple injection of the aqueous solution into the polymer solution. The amount of polymer in the acetone solution was determined by the requested N/P ratio. The resulting mixture further was slowly injected into 10 ml of a magnetically stirred 0.1 % aqueous Pluronic™ solution, using a 14-gauge needle. Under these conditions spontaneous nanoparticle formation could be observed. The nanoparticle preparation was stirred for 3 hours under constant laminar air-flow for complete removal of residual acetone. Particles were characterized and used directly after the preparation.

Particle Size Measurement

The effective hydrodynamic diameter was measured by photon correlation spectroscopy (PCS) using a Malvern Zetasizer 4 (Malvern Instruments, Germany) at 25°C equipped with a 5 mW helium neon laser and the Malvern software. Samples were measured in a AZ 110 cell at 633 nm and a scattering angle of 90°. The samples were diluted in ultrapure water if needed, to measure within a defined count rate interval of 100 – 400 kilo counts / second to avoid multiscattering. The viscosity (0.88 mPa s) and the refractive index of ultrapure

water (1.33) were used for data analysis. The PCS V. 1.26 - software was used to calculate particle mean diameter and width of fitted gaussian distribution. All measurements were carried out in triplicate.

Zeta Potential Measurements

ξ-potential measurements were carried out using the Zetasizer 4 (Malvern Instruments, Germany). Samples were diluted in ultrapure water to a defined count rate interval of 400 – 800 kcps. Electrophoretic light scattering was performed in a AZ 104 cell. Average ξ-potential values were calculated from the data of 3 runs. The instrument was calibrated with a Malvern –50 mV transfer standard. All measurements were carried out directly after particle preparation.

Polymer Mass Balance

The polymer mass balance was determined gravimetrically after centrifugation of the nanoparticle preparation directly after preparation at 10,000 rpm (8,176 g) in an Eppendorf 5415C centrifuge for 20 min. The nanoparticle pellet and the supernatant were lyophilized separately in a Christ beta-II freeze-dryer (Osterode, Germany). The polymer mass balance was measured gravimetrically to rule out the possibility of free polymer in solution, taking into account the 0.1 % poloxamer stabilizer. The difference of all population means was analyzed by a two-sample t-test and one-way ANOVA at the 0.05 level.

Atomic Force Microscopy

The DNA nanoparticles were directly transferred onto a silicon chip after preparation, by dipping into the nanoparticle solution. Atomic force microscopy was performed on a Digital Nanoscope IV Bioscope (Veeco Instruments, Santa Barbara, CA) as described elsewhere [15]. The microscope was vibration-damped. Commercial pyramidal Si_3N_4 tips (NCH-W, Veeco Instruments, Santa Barbara, CA) on a cantilever with a length of 125 μm, a resonance frequency of

about 220 kHz and a nominal force constant of 36N/m were used. All measurements were performed in Tapping mode™ to avoid damage of the sample surface. The scan speed was proportional to the scan size and the scan frequency was between 0.5 and 1.5 Hz. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction, and the height signal in the retrace direction, both signals being simultaneously recorded. The results were visualized either in height or in amplitude modus.

Lactate Dehydrogenase Release

The release of lactate dehydrogenase (LDH) was measured to characterize the membrane toxicity of nanoparticle formulations. L929 mouse fibroblasts (DSMZ, Braunschweig, Germany) were seeded at a density of 50,000 cells per 2 ml in 12 well culture dishes (Nunc, Wiesbaden, Germany) and grown for 24 h prior to the incubation with the particles, according to the supplier's recommendations. The cells were washed twice with PBS buffer (0.1 M, pH 7.4) and incubated with 100 µl nanoparticle suspension resulting in 0.5 mg polymer per ml PBS buffer. Blank PBS buffer and a 0.1 % Triton-X 100 solution in PBS buffer were used as controls. 100 µl samples were withdrawn after 120 and 180 min and processed according to the manufacturer's instructions (Sigma Diagnostics, Deisenhofen, Germany). All sample values were normalized relative to Triton-X values and expressed as relative LDH release in [%]. All DEAPA polymers were used as nanoparticle preparations at N/P ratio of 5. Each sample was performed in triplicate. The difference of all population means were analyzed by a two-sample t-test and one-way ANOVA at the 0.05 level.

DNA Release and DNase Stability

DNA release from the nanoparticles was evaluated in the supernatant of the particle preparations in isotonic TE-buffer at pH 7.4 (1 mM Na_2EDTA; 10 mM Tris; 143 mM NaCl). Aliquots containing 300 µl of the nanoparticle suspension

corresponding to 7.5 µg pDNA were prepared for each time point. Polymer mass was dependent on the N/P ratio. After predetermined time points, the samples were centrifuged at 14000 rpm (16,025 g) for 30 min in an Eppendorf 5415C centrifuge. The supernatant and the pellet were lyophilized in a Christ beta-II freeze-dryer (Osterode, Germany). The soluble components from the supernatant were re-dissolved in 20 µl TBE-buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA), containing 25 IU heparin (Serva, Heidelberg, Germany) to separate possible DNA / backbone complexes. Glycerol (5 µl) was added to the preparation prior to the separation using a 1% agarose gel.

DNA stability was studied using 100 µl aliquots of the nanoparticle suspensions, corresponding to 2.5 µg DNA. The samples were incubated with 12.25 µl DNase buffer 10x (1M Na-acetate, 50 mM $MgCl_2$) and 2.5 µl DNase I solution (DNase I, Boehringer Mannheim, Germany) (50 I.U. / ml in 50 mM Tris-HCl pH 8, 100 mM KCl). The reaction was terminated with 5.7 µl EDTA solution (0.5 M, pH 8). The probes were freeze-dried and stored at –20 °C until further use. At the time of analysis, the dried probes were incubated for one hour in 10 µl TBE-buffer containing 50 I.U heparin. Further, 10 µl Roti-phenol® (Roth, Karlsruhe, Germany) were added and incubated for additional 2 hours at room temperature. Glycerol (5 µl) was added to the emulsion before application onto a 1 % agarose gel. Untreated DNA was applied to the gel for comparison of the DNA forms. Electrophoresis (Blue Marine 200, Serva, Germany) was carried out at 100 V for two hours in TBE-buffer. 8 µl 1 % (w/v) ethidium bromide solution were included into all gels to visualize the DNA localization by photography with UV transillumination.

Nanoparticle Cell Association

Flow cytometry was performed using plasmid DNA labeled with 25 µl/mg DNA of the intercalating fluorescence dye YOYO-1 (Molecular Probes, Leiden, The Netherlands), as described by Ogris et al. [16]. L929 mouse fibroblasts were

plated at a density of 400, 000 cells / well in 6-well cell culture dishes (Nunc, Wiesbaden, Germany) and grown for 24 hours in DMEM with 10 % FCS. Nanoparticles were prepared with the polymer P(68)-10 and the fluorescence labeled DNA, using the standard protocol at different N/P ratios. The volume of the nanoparticle dispersion was reduced by ¼ to 2.5 ml. Aliquots of 160 µl particle suspension, containing 4 µg DNA were incubated with the cells for 4 hours according to the transfection protocol.

The cells were washed twice with glucose 5 %, pH 7.4 and once with 1M NaCl as described by Ruponen et al. to remove adsorbed nanoparticles [17]. The cells were suspended in PBS buffer after detachment by trypsin incubation for 1 min. Cell suspensions were kept on ice until analysis. Flow cytometry was performed with 10,000 cells, using a Becton Dickinson FACS Scan equipped with an argon laser with an excitation wavelength of 488 nm.

Cellular uptake of DNA Nanoparticles

For confocal microscopy experiments a Zeiss Axiovert 100M microscope coupled to a Zeiss LSM 510 scan module was used.

Plasmid DNA was covalently fluorescence labeled with a rhodamin dye, Cy-3 (Mirus, Madison, Wisconsin) according to the manufacturers instructions. The P(68)-10 amine-modified polyester was covalently labeled with a fluorescein chromophore using the amine reactive 5-DTAF (5-(4,6-dichlorotriazinyl)aminofluorescein, Molecular Probes, Leiden, The Netherlands). For polymer labeling, 96 mg of the polymer and 2.5 mg 5-DTAF were dissolved in a total volume of 7.5 ml DMSO and stirred for 1.5 hours at 65°C for amine coupling. The polymer was ice-cooled and precipitated with a mixture of propanol-2 and water. The pellet was washed several times to eliminate unbound components.

Nanoparticles were prepared according to the standard protocol by mixing the fluorescence labeled polymer with non-labeled polymer in a mass ratio of 1/6.

Rhodamin labeled DNA was mixed with the original plasmid in a ratio of 1/61.5. L929 cells were seeded at a density of 20,000 cells per well in 8 well chamber slides (Lab Tek, Nunc, Wiesbaden, Germany). After 24 hours medium was removed. Aliquots of the nanoparticle suspension containing 0.8 µg DNA were added to new medium containing 10 % FCS. The cells were incubated for 5, 30, 60 and 180 minutes with the nanoparticles. The medium was removed and cells were washed 4 times with PBS buffer. Fixation of cells was performed by incubation with 400 µl paraformaldehyde solution 3 % in PBS for 20 minutes. The cells were washed again for 4 times with PBS and incubated for additional 20 minutes with a 0.1 mg/ml DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Molecular Probes, Leiden, The Netherlands) in PBS for nucleus identification. An Enterprise UV laser with a wavelength 364 nm was used for excitation of the blue DAPI fluorescence. Excitation of green fluorescence of 5-DTAF labeled polymer was performed using an argon laser with an excitation wavelength of 488 nm. A Helium-Neon laser with an excitation wavelength of 543 nm was used and for the excitation of red fluorescence of the DNA. Images were recorded in multitracking mode using a longpass filter of 385 nm for DAPI, a longpass filter of 505 nm for Oregon Green and a longpass filter of 560 nm for rhodamine.

Transfection Efficiency

L929 mouse fibroblasts (DSMZ, Braunschweig, Germany) and NIH-3T3 fibroblasts (DSMZ, Braunschweig, Germany) were cultured in Dulbeco's modified Eagle’s medium (DMEM, Gibco, Eggenstein, Germany) and supplemented with 10 % fetal calf serum (FCS, Gibco) according to the supplier’s recommendations. The cells were plated 24 h before nanoparticle incubation at a density of 50,000 cells / 2 ml in 12 well plates (Nunc, Wiesbaden, Germany). U937, human pre-monocytic cells (DSMZ, Braunschweig, Germany), cultured according to the supplier’s instructions, were

plated at a density of 50,000 cells / 2 ml in RPMI medium containing 10 % FCS and incubated for 72 h with 81 nmol / ml phorbol 12-myristate 13-acetate (PMA, Sigma, Deisenhofen, Germany) for cell activation and adhesion to the cell culture dish. Rabbit vascular smooth muscle cells (RbVSMC), a primary cell line was supplied by the department of experimental radiology (University of Marburg) after their isolation according to Axel et al. [18]. The cells were cultured in DMEM medium supplemented with 10 % FCS. The RbVSM cells were plated at a density of 20,000 cells / 2 ml because of their larger cell size. Immediately prior to transfection, the medium was removed and replaced by 500 µl glucose 5 % at pH 7.4. Aliquots of 160 µl particle suspension, containing 4 µg pLuc-CMV, were added to the glucose 5 % medium at pH 7.4.

The cells were pre-incubated with the nanoparticle suspension for 5 min, after which 1.5 ml cell culture medium containing 10 % FCS was added. The nanoparticle suspension was dispersed in the glucose medium before the addition of the medium, since instabilities of nanoparticle suspension were observed in the medium. The nanoparticle suspension was removed after 4 hours of incubation and replaced with fresh medium containing 10 % serum. Cells were harvested after 48 h, washed with PBS pH 7.4 twice, and lysed in cell culture lysis reagent (Promega). Luciferase content was assessed using a commercial luminescence kit (Promega) measured in a Berthold Sirius luminometer (Berthold, Pforzheim, Germany). RLUs were converted into luciferase content by calibration with recombinant luciferase (Promega). Protein concentrations were determined by a modified BCA assay [19]. Transfection experiments were performed in triplicate and presented as the mean of the luciferase / protein ratio [ng/mg].

RESULTS

Particle Preparation and Characterization

DEAPA (P), DEAEA (E) and DMAPA (M) -modified PVA-graft-polyesters, represented in Figure 1, were used in this study for DNA nanoparticle formation.

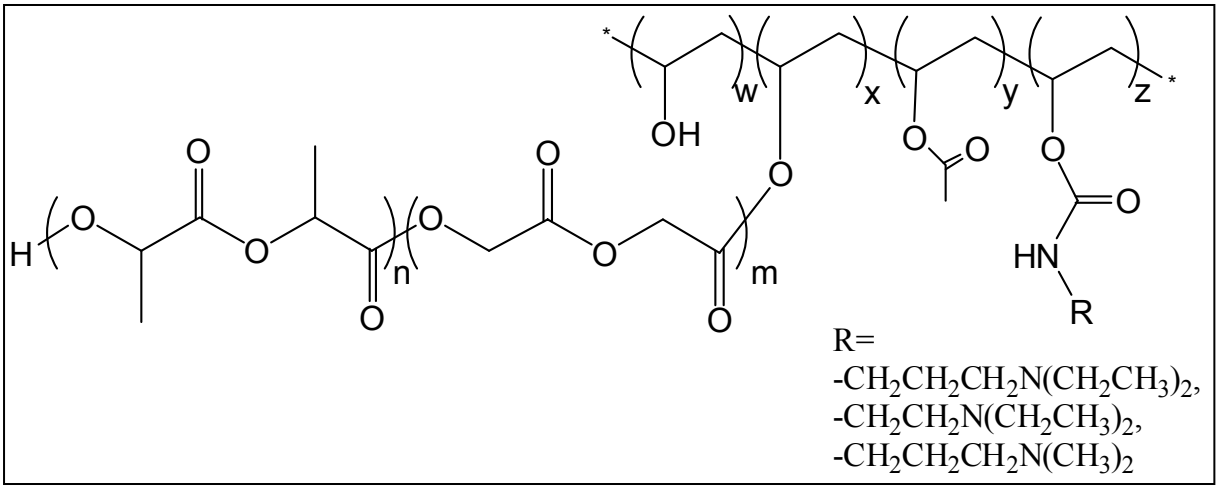

Fig.1: *Representative structure of the biodegradable amine-modified (polyvinyl alcohol)-graft-poly(lactide-co-glycolide) polymers. The PVA degree of polymerization was 300 containing from 12 to 68 amine-substituents and 150 to 240 grafted PLGA side chains. The PLGA (50:50) grafts were built up of approximately 10 or 20 monomer units.*

The nanoparticles were formulated using variable nitrogen to phosphate (N/P) ratios, based on the fact that one amine side chain represented one protonable amine. The amount of DNA used in our study was held constant at 250 µg DNA for each preparation. The properties of the branched polyesters allowed us to modify the solvent displacement method for optimized DNA encapsulation.

This was highlighted by the fact that, although DNA (0.5 mg/ml) was not soluble in acetone, no precipitation occurred after the addition of 500 µl of the

DNA solution into the acetone polymer solution (2.5 ml). In contrast, the addition of DNA solution into an acetone solution resulted in a visible precipitation. Nanoparticles formed spontaneously after the injection of the acetone/water solution containing DNA and the polymer into the aqueous stabilizer medium.

Polymer	**Size** [a] **[nm]**	**Poly-dispersity** [a]	**ξ - potential** [a] **[mV]**	**Molecular Weight** [b] **[g/mol]**
P(12)-10	238.0	0.25	24.2 ± 1.7	262,600
P(26)-10	199.4	0.28	46.7 ± 0.7	n.d.
P(33)-10	175,4	0.53	45.4 ± 0.4	366,900
P(68)-10	280.5	0.4	45.6 ± 0.4	798,500
P(33)-20	285.1	0.25	45.0 ± 1.0	711,900
E(33)-10	211.7	0.32	44.7 ± 1.5	1199,000
E(12)-20	188.7	0.68	41.3 ± 0.6	350,300
E(33)-20	> 1000	1.0	n.d.	767,000
M(13)-10	186.7	0.31	38.9 ± 1.2	631,700
RG 502H	563.4	0.74	-54.6 ± 2.6	15,200

[a] Average value of three independent measurements and standard deviation

[b] MW from GPC-MALLS (gel permeation chromatography - multiple-angle-laser-light-scattering) according to Wittmar et al. [13]

Table 1: *Characterization of DNA nanoparticles prepared with amine-modified PVA - graft polyesters at a N/P ratio of 5 and one PLGA (RG 502H) polymer, using a modified solvent displacement method. The nanoparticles were characterized directly after their preparation with regard to their hydrodynamic diameters by PCS and their ξ - potentials by electrophoretic light scattering. The molecular weights of the of the polymers were specified in the table.*

Particles sizes and ξ-potentials were measured directly after preparation and results are presented in Table 1. DEAPA polymer nanoparticles at the N/P ratio of 5 had hydrodynamic diameters ranging from 175 – 285 nm, while DEAEA nanoparticle sizes were in the range of 200 nm. The E(33)-20 polymer did not form nanoparticles at the N/P ratio investigated in this study. The DMAPA polyester studied, M(13)-10, had an average hydrodynamic diameter of 187 nm. Particle sizes were independent of the polyester side chain length and amine-modification. However, the commercial PLGA (RG 502H) polymer particles showed roughly a two-fold increase in hydrodynamic diameter as compared to the amine-modified polyester formulations when the same method of particle formation was used.

The ξ-potential of the DNA / PLGA nanoparticle preparation was highly negative, arising from the presence of DNA and the uncapped carboxylic groups of the polymer. The DNA nanoparticles formulated with the amine-modified polyesters had very similar positive ξ-potentials, independent of the polymer used, when they were prepared at the N/P ratio of 5.

In Figure 2 nanoparticles prepared with P(68)-10, as a representative polymer, at different N/P ratios were studied for their size and ξ-potential. Nanoparticles with HT DNA at a N/P ratio of 0.5 were relatively large, measuring approximately 890 nm. At the calculated point of neutrality, the nanoparticle formulation exhibited a mean hydrodynamic diameter of approximately 200 nm. Further increase in the N/P ratio did not have any influence on the nanoparticle size measured. The polymer P(68)-10 was further used to study the effect of the N/P ratio on the nanoparticle ξ-potentials (Fig.2).

At the N/P ratio of 0.5, the particles exhibited a negative ξ-potential (- 48.6 mV). From the point of calculated charge neutrality on, the ξ-potentials increased to reach a constant level at N/P 3 to N/P 9 (+ 51 mV) (Fig.2).

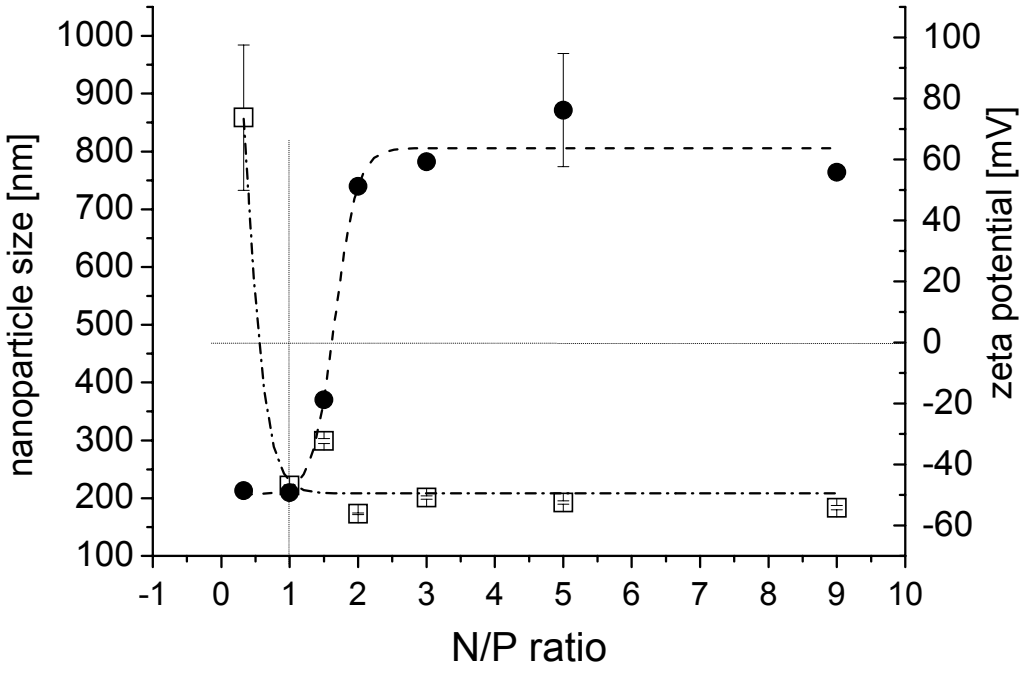

Fig.2: *Nanoparticle size (□) and ξ-potential (●) of DNA nanoparticles prepared with the amine-modified polymer P(68)-10 at different N/P ratios. The nanoparticle size remains approximately constant and small from N/P 1 to N/P 9, whereas the ξ-potential increases from N/P 1 to N/P 3 by the value of 100 mV.*

Polymer Mass Balance

The amphiphilic properties of the polymers observed during particle preparation were attributed to the hydrophilic amine modifications and the remaining hydroxyl groups of the PVA backbone combined with the short hydrophobic PLGA grafted side chains.

As a result, it was necessary to investigate if the polymer molecules could solubilize in the aqueous stabilizer solution. The mass balance of the nanoparticle suspension compared to the polymer mass in the supernatant was

characterized. Gravimetric analysis of the lyophilized supernatants and of the pellets added up to the total yield.

The total yield was > 81% for the nanoparticle suspensions characterized in this study (Table 2). Small polymer amounts (1.61 – 5.6%) were recovered in the supernatant, suggesting that the amphiphilic properties of the polymer did not result in the solubilization of the polymer in the aqueous medium after particle preparation. A high amine substitution, and thus theoretically a more hydrophilic polymer resulted in an almost complete recovery of the polymer mass in the pellet.

Polymer	**Pellet [%]±sd**	**Supernatant [%]±sd**	**Recovered Mass [%]±sd**
P(12)-10	97.11 ± 1.79	2.89 ± 1.79	81.33 ± 3.06
P(26)-10	97.11 ± 2.51	2.89 ± 2.51	88.00 ± 3.27
P(33)-10	96.26 ± 5.77	3.74 ± 5.77	94.00 ± 1.06
M(13)-10	96.15 ± 2.81	3.85 ± 2.81	91.73 ± 1.15
E(33)-10	98.39 ± 2.45	1.61 ± 2.45	101.87 ± 2.05
P(33)-20	97.66 ± 2.72	2.34 ± 2.72	90.00 ± 11.00
RG 502H	94.40 ± 4.85	5.60 ± 4.85	100.00 ± 14.90

Table 2: *Polymer mass balance of the recovered mass of the DNA nanoparticle preparations at N/P ratio of 9. The recovered mass [%] was in the range of 80 – 100% for the amine-modified polyesters as well as PLGA. The mass fraction recovered in the pellet was approximately 95% of the recovered mass for all preparation. At the 0,05 level, the difference of all population means were not significantly different from the PLGA (RG 502H) nanoparticles analyzed be two-sample t-test and one-way ANOVA.*

Atomic Force Microscopy

Atomic force microscopy (AFM) was used to characterize the morphology of the nanoparticles (Fig.3). The particle sizes measured by dynamic light scattering could be confirmed by the micrographs.

The DNA / P(26)-10 polymer particles were distinct and spherical.

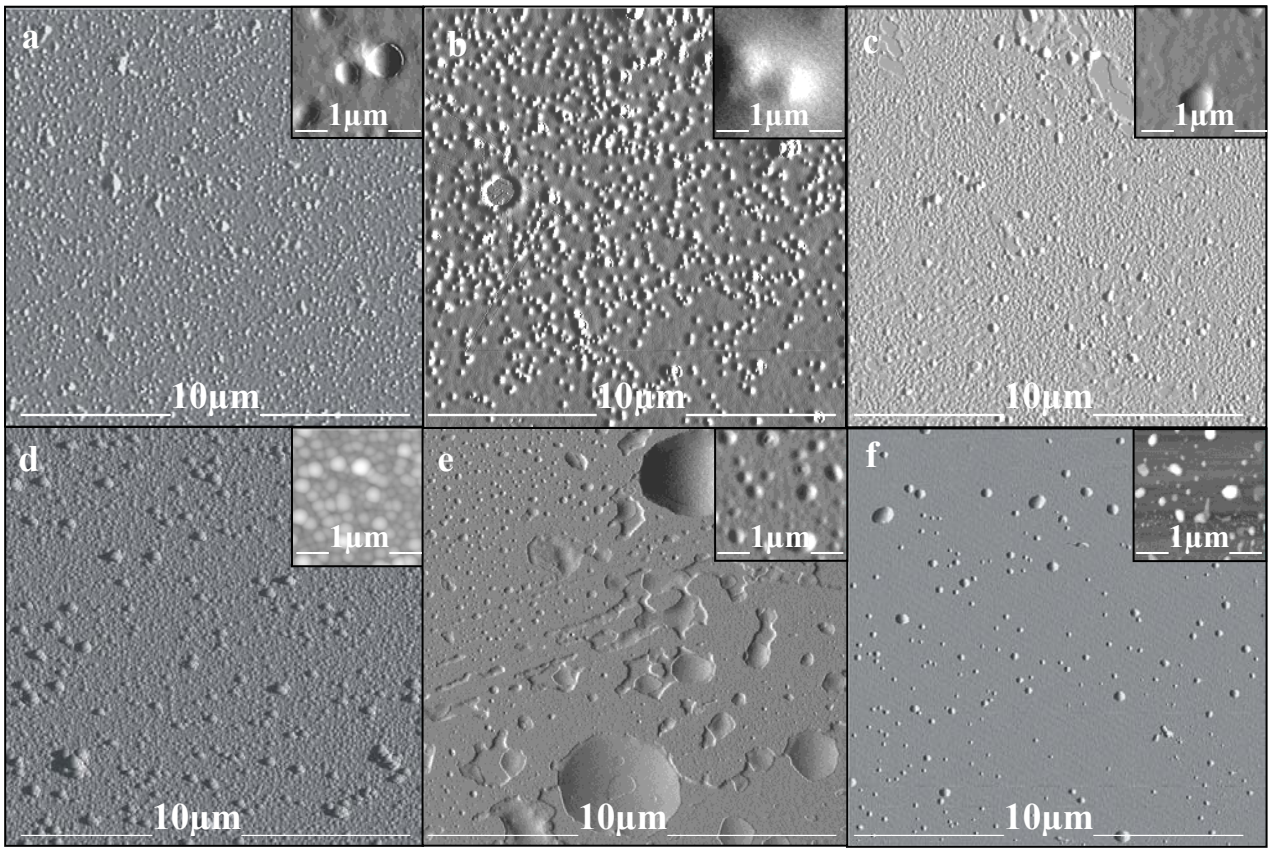

Fig.3: *AFM imaging of DNA nanoparticles preparations: P(26)-10 N/P 9 (a), P(33)-10 N/P 11 (b), P(68)-10 N/P 5 (c), M(13)-10 N/P 5 (d), E(33)-10 N/P 5 (e), RG 502H (f). Nanoparticles have well defined structures and could confirm the particle size measurements. E(33)-10 nanoparticles led to a more collapsed structure on the silicium support.*

Nanoparticles prepared using E(33)-10 led to larger particles which had a less discrete morphology up to a collapsed structures. DMAPA particles, used in a N/P ratio of 5, as well as the PLGA nanoparticles were again round and well defined.

LDH - Release

The amounts of LDH released from cells incubated with the nanoparticles of the homologous series of the DEAPA polymers at the N/P ratio of 5 did not show significant differences analyzed by a two-side t-test and one-way ANOVA ($P \leq 0.5$) (Fig.4a).

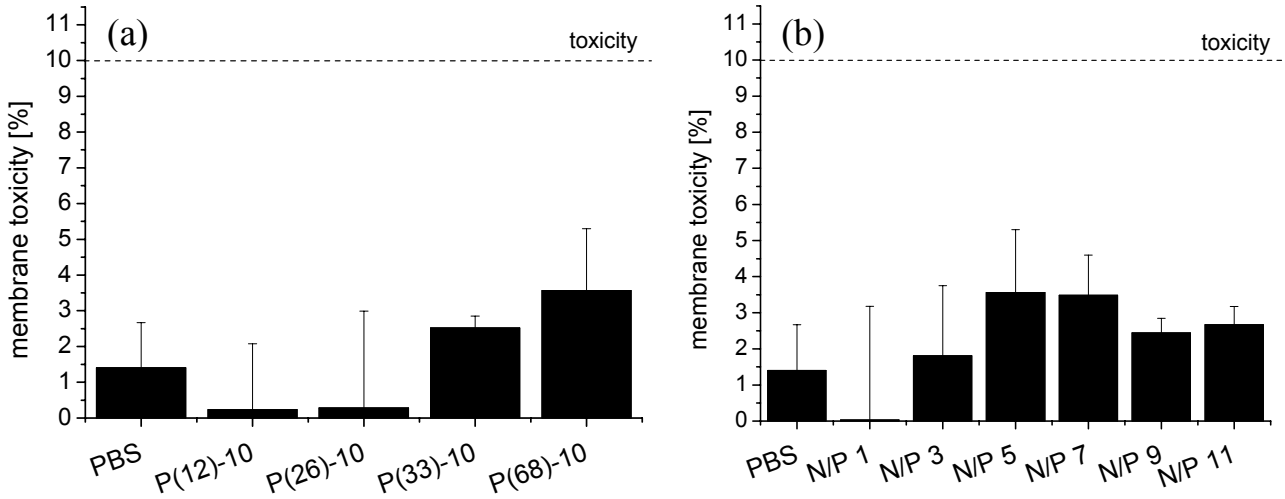

Fig. 4: *LDH-release assay after 3 hour incubation of 1 mg nanoparticles in PBS buffer, (a) homologous series of DEAPA substituted polyesters all at N/P ratio of 5; (b) DNA P(68)-10 nanoparticles at different N/P ratios. At the 0.05 level, the difference of all population means were not significantly different from the blank PBS medium analyzed by two-sample t-test and one-way ANOVA.*

Further, the effect of the N/P ratio (1 to 13) on the membrane stability was studied, using P(68)-10 polymer nanoparticles (Fig.4b). No membrane toxicity over the 10 % level was detected taking into account that no significant differences compared to the PBS blank were observed using the statistical analysis two-side t-test and one way ANOVA test ($P \leq 0.05$).

DNA Release and Enzyme Stability

DNA release from the nanoparticles, prepared at the N/P ratio of 5, was studied over 9 days in isotonic TE buffer, pH 7.4 using separate aliquots for each time point which contained 7.5 μg DNA (Fig.5).

Agarose gel electrophoresis of the supernatants of the P(12)-10 and the PLGA particles showed high levels of DNA in the supernatant from day 0 on, implying that the encapsulation efficiency of these preparations was incomplete compared to the other amine-modified polymer particles.

In both the DNA / P(33)-10 and the DNA / P(68)-10 nanoparticle supernatants no DNA was apparent in the gel directly after the nanoparticle preparation, demonstrating complete DNA retention in the formulation. In the supernatant of P(33)-10 no DNA release was observed within the 9 days of incubation, whereas P(68)-10 polymer nanoparticles released small amounts of DNA beginning from day 2 on.

The stabilizing effect of the nanoparticles against DNA degradation by DNase was studied with P(68)-10 particles (Fig.6). Nicking of naked DNA took place by the conversion of the supercoiled form into the open circular form and ended in the complete destruction of the DNA. Formulations at N/P ratio of 0.5, did not offer much protection, as the DNA was completely degraded after 5 minutes of enzyme incubation.

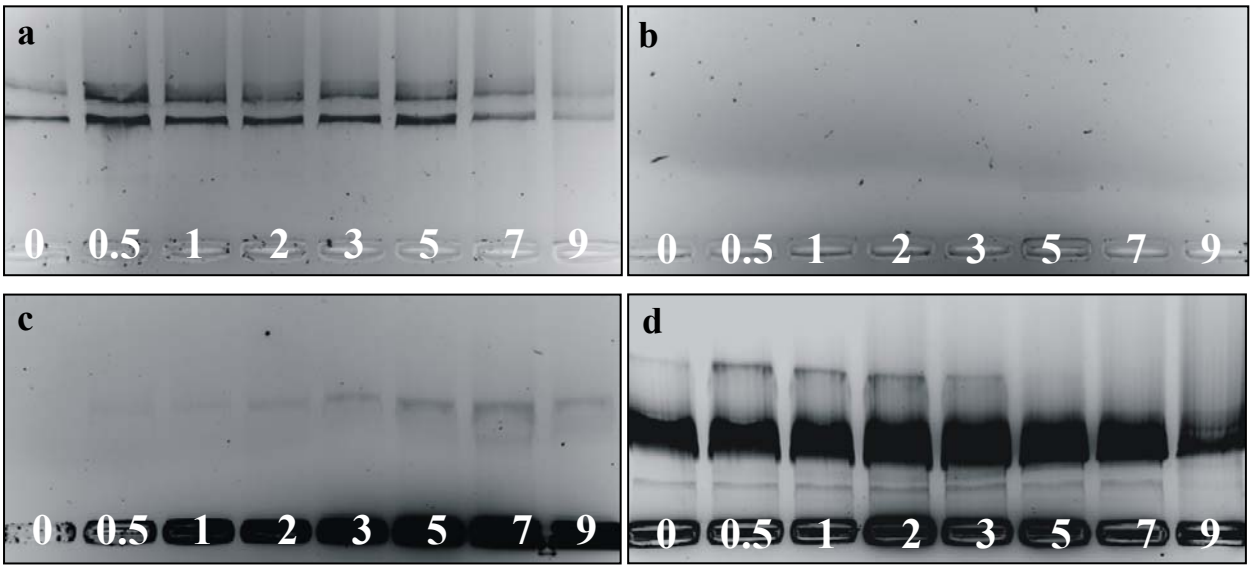

Fig.5: *Agarose gel of DNA release from nanoparticles at N/P 5 from 0 to 9 days at pH 7.4 from the supernatant, a) P(12)-10; b) P(33)-10; c) P(68)-10; d) PLGA. Complete DNA encapsulation could be demonstrated for the P(33)-10 and P(68)-10 nanoparticles.*

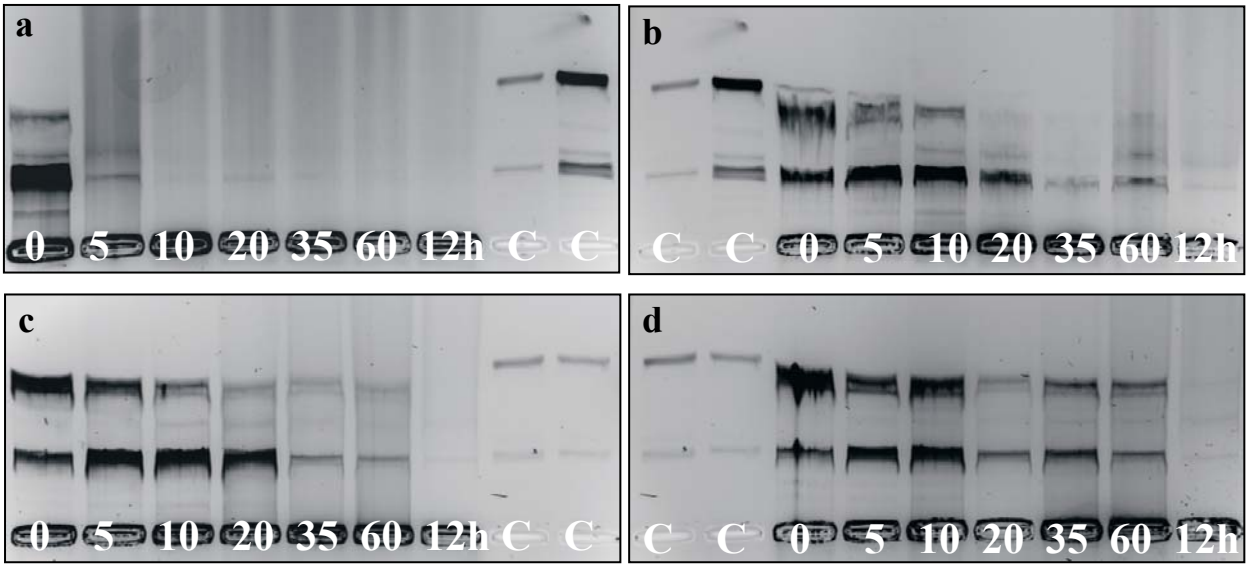

Fig.6: *DNA protection from enzyme degradation by encapsulation in P(68)-10 nanoparticles, followed from 0 minutes to 60 minutes and 12 hours of incubation with DNase I a) N/P 0.5; b) N/P 1; c) N/P 3; d) N/P 5. C represents non-degraded DNA samples.*

In contrast, protection of DNA could be observed in formulations at the N/P ratio of 1 for 35 min, while for N/P 3 and N/P 5 the DNA was only degraded after 12 hours. Nicking of the supercoiled DNA-form could be observed in the N/P 3 preparation after 60 min of incubation. The DNA of the N/P ratio of 5 particles was at the same time point still present in the supercoiled form.

Nanoparticle Cell Association

Nanoparticles prepared with raising N/P ratios were found to associate better with the L929 fibroblast cells as shown in Figure 7 and by the geometric means of the fluorescence counts.

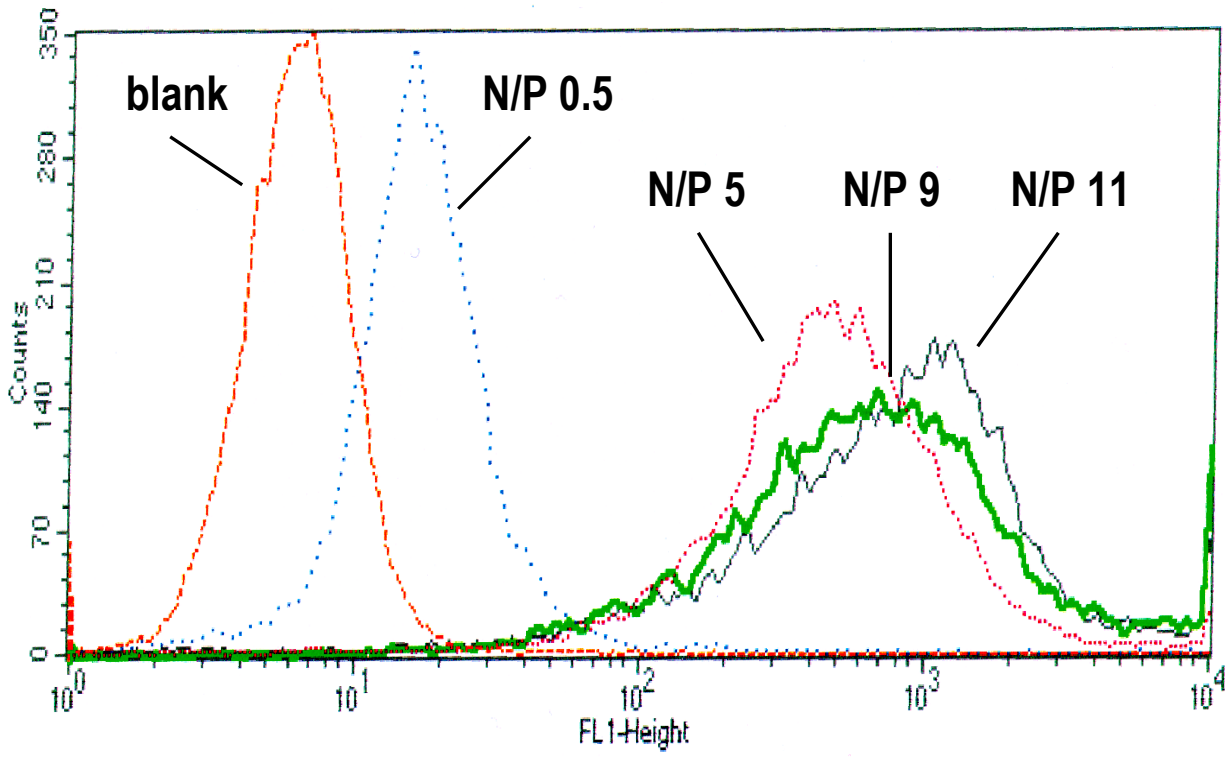

Fig.7: *YoYo - 1 fluorescence labeled DNA association with cells after incubation of DNA / P(68)-10 nanoparticles at different N/P ratios. DNA association with the cells increases with the N/P ratio. Nanoparticles formulated at N/P 0.5 exhibited a minor association. Geometric means (gm) of the fluorescence, blank (6), N/P 0.5 (18), N/P 5 (430), N/P 9 (569), N/P 11(619).*

Intensive washing with glucose 5% and high ionic salt solution reduced the adsorption of the nanoparticles on the cell surfaces. Therefore, the fluorescence measurement can be in majority ascribed to nanoparticle uptake via non-specific nanoparticle endocytosis. For nanoparticle formulations of a N/P ratio of 5 the DNA uptake was extensively increased (gm: 430) compared to blank cells (gm: 6) and to cells incubated with nanoparticles formulated at a N/P ratio of < 1 (gm:18). A doubling of the calculated positive charge excess, represented by particles prepared at a N/P ratio of 9 (gm: 569) and 11 (gm: 619), exhibited only a slight increase in fluorescence counts per cell.

Cellular uptake of DNA Nanoparticles

The double labeling enabled us to simultaneously follow the transport of DNA and the polymer P(68)-10 into mouse fibroblasts in vitro (Fig.7). After a 5 minute incubation only little fluorescence of the particles was associated to the cells or the cell membranes. Compared to that, the 30 minutes time-point exhibited cells that already had taken up nanoparticles. The fluorescence was localized in several defined areas of the cells. The red, rhodamine fluorescence of the DNA and the green P(68)-10 fluorescence were co-localized. At the 1 hour time-point the fluorescence was localized in large vesicles and remained superimposed. The three hour time-point exhibited cells that had endocytosed very large amounts of DNA nanoparticles. These were not arranged in groups anymore but dispersed over the entire cell and specifically around the nucleus. Fluorescence of the DNA and polymer remained superimposed, whereas diffuse polymer fluorescence was detectable throughout the cytoplasm. The red DNA fluorescence remained concentrated in specific areas.

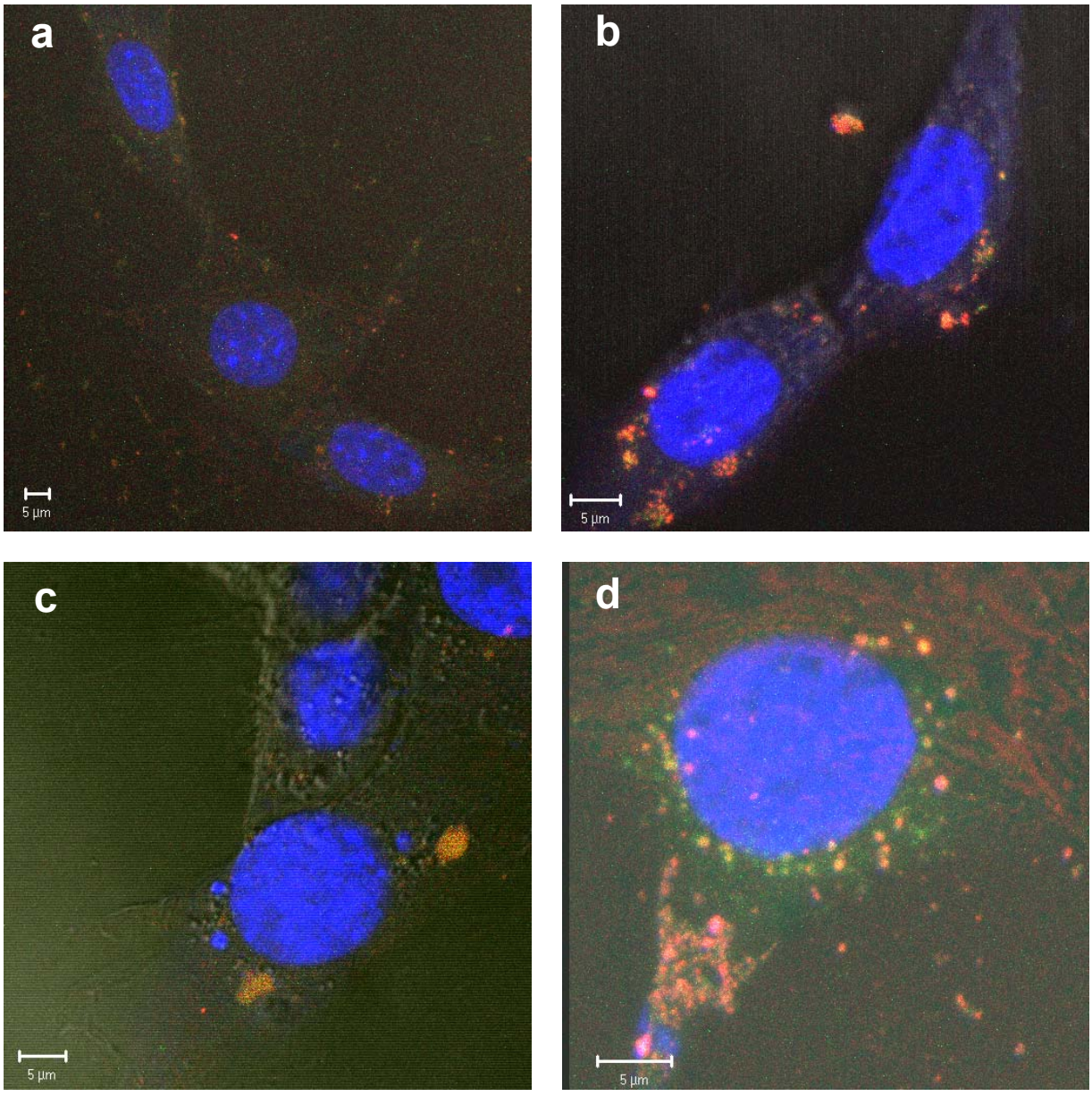

Fig.8: *Confocal laser scanning microscopy micrographs of DNA nanoparticle uptake at different time-points. (a) 5 min, (b) 30 min, (c) 60 min, (d) 180 min. The nanoparticles are extensively taken up into the endosomal compartment of the cells from 30 min post incubation on. DNA (red) and the P(68)-10 polyester (green) were associated during incubation and uptake, represented by the yellow/ white fluorescence.*

Transfection Efficiency

The transfection efficiency of DNA nanoparticles prepared with the homologous series of DEAPA and DEAEA polymers were investigated in L929 mouse fibroblasts (Fig.9-10). All DNA nanoparticles were prepared at a N/P ratio of 9 by adjusting the polymer mass during formulation using invariant amounts of plasmid DNA. Hence, equal volumes of particle suspension were added to the cells, each containing 4 µg of plasmid DNA per well.

The transfection efficiency of the nanoparticle suspension was evaluated and compared to that of an equal amount of free DNA in solution, to DNA/PEI 25 kDa complexes and a DNA / PLGA (RG 502H) nanoparticle suspension. The luciferase expression of cells incubated with most of the amine-modified polymer DNA nanoparticles was greater than that of naked DNA. The efficacy of the DEAPA polymers to transfect increased with the degree of amine modification of the polymer, resulting a maximum value of luciferase expression for the P(68)-10 polymer (Fig.9a). P(68)-10 nanoparticles exhibited a 40,950-fold higher transfection efficiency than DNA in solution and an 8.57-fold higher luciferase expression than DNA/PEI complexes, both prepared at N/P 9. PLGA nanoparticles achieved only slight luciferase expression. Polyesters with reduced amine modification as well as those with 20 units of PLGA grafting were less effective than P(68)-10. The polyester, P(68)-10, in consequence, was investigated more intensively. P(68)-10 / DNA nanoparticles were formulated at N/P 0.5 to N/P 11 and their transfection efficiencies are represented in Figure 9b. The values were compared to DNA in solution and DNA/PEI complexes at N/P 5. The amount of luciferase increased exponentially from N/P 0.5 to N/P 7. A plateau was reached at N/P 7 through to N/P 11. Nanoparticles at N/P 0.5, which exhibited a negative charge, nonetheless, achived higher transfection efficiencies than DNA in solution. At an equal N/P ratio (N/P 5), the nanoparticles were 273-times more effective than the PEI/DNA complexes (Fig.9b).

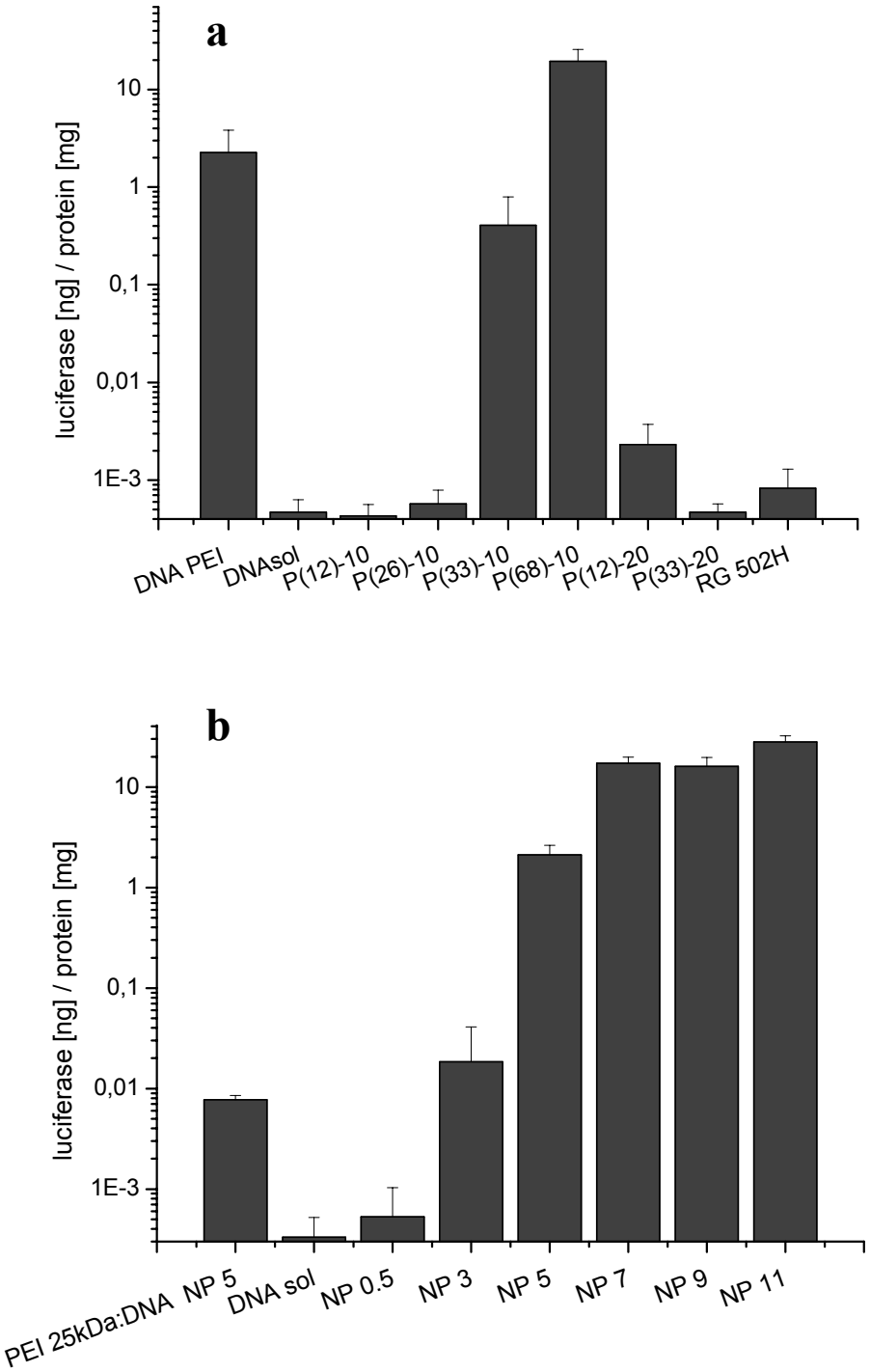

Fig.9: *Transfection efficiencies of DNA DEAPA nanoparticles, a) homologous series of DEAPA substituted polyesters and PEI 25kDa / DNA complexes all at N/P 9 compared to DNA in solution and DNA PLGA nanoparticles, b) DNA P(68)-10 nanoparticles at different N/P ratios and PEI 25kDa at N/P 5.*

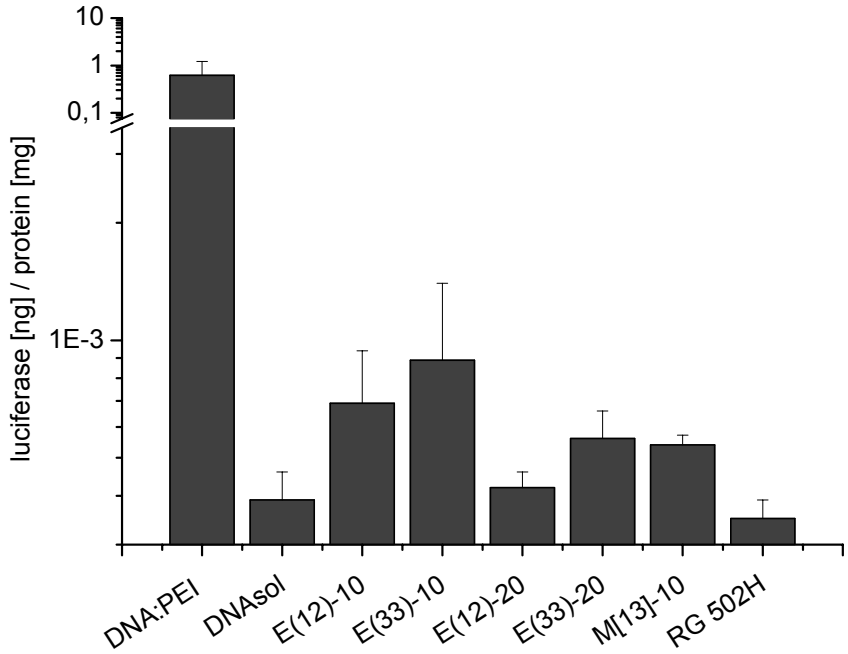

Fig.10: *Transfection efficiencies of DNA DEAEA nanoparticles, homologous series of DEAEA substituted polyesters and PEI 25kDa / DNA complexes all at N/P 9 compared to DNA in solution and DNA PLGA nanoparticles. The higher amine-modifications result in higher transfection. The values however are inferior to PEI25 kDa / DNA complexes.*

The DEAEA polyester nanoparticles were also able to transfect L929 cells, however, DEAEA polymers were not as efficient as the DEAPA series (Fig.10). DNA/PEI complexes exhibited a nearly 700-fold better transfection efficiency than the most potent nanoparticles (E(33)-10), at N/P 9. The E(33)-10 DNA nanoparticles showed only a 2.3-fold higher efficiency than plasmid DNA in solution. The only DMAPA polymer used in this study exhibited a minimal increased transfection efficiency compared to DNA in solution (Fig.10).

The transfection efficiency of the nanoparticles at a N/P ratio of 9 was also investigated with three complementary cell lines: i) PMA activated U937 human pre-monocytic cell line, ii) rabbit smooth muscle cells (RbVSM) and iii) NIH-3T3 mouse fibroblasts. The results of the P(33)-10, P(68)-10 and PEI 25 kDa were compared to those of DNA in solution (Table 3). It was demonstrated that the transfection efficiency was greatly enhanced for the P(33)-10 and P(68)-10 polyesters compared to free DNA in non-monocytic cells.

Cell line	PEI/DNA	P(33)-10	P(68)-10	DNA sol.
U937	1.6	1.5	1.8	1
L929	4,545.5	818.2	39,090.9	1
RbSMC	1,470.6	132.3	1,147.1	1
NIH – 3T3	10,989.0	604.4	17,582.4	1

Table 3: *Effect of the cell line on the transfection efficiency of DNA : DEAPA nanoparticles and DNA/PEI 25 kDa complexes at N/P 9 compared to free DNA. The DNA / P(68)-10 nanoparticles exhibit similar efficiency than the PEI 25kDa / DNA complex*

Monocytic cells, however, did not exhibit luciferase expression. The P(68)-10 formulation was more efficient than the DNA/PEI complex in fibroblasts. The measured values were reduced for all the preparations in RbVSM, where the PEI / DNA complex was 1.3-fold more efficient than the P(68)-10 nanoparticles. P(33)-10 particles consistently exhibited lower luciferase levels than P(68)-10 and PEI 25 kDa but still the values were greatly enhanced compared to DNA in solution.

DISCUSSION

A tremendous effort has been put into the development of new delivery devices for gene delivery and more particularly into the development of new formulations for the effective use of DNA as a vaccine. In this study we investigated a new polymer class to evaluate its efficacy of DNA encapsulation, gene transfer and conservation of DNA bioactivity after nanoencapsulation. The polymers were designed specifically for DNA delivery by combining different functional modules which, from our hypothesis, were favorable for DNA encapsulation, in a polymer (e.g. biodegradation, hydrophobic PLGA grafts and cationic, hydrophilic amine substitutions) [13]. These possible structural varieties resulted in a spectrum of characteristics that were differently pronounced depending on the proportions in the polymer composition.

The encapsulation of hydrophilic molecules in hydrophobic biodegradable polymers has been a challenge for some time. It was previously accomplished using ternary systems, such as emulsification / solvent evaporation techniques and double emulsion encapsulation techniques [9]. All of these methods, however, used high energy sources to stabilize the molecule in the polymer matrix [8,11,20]. The solvent displacement method is typically not an efficient technique for the direct encapsulation of water soluble drugs [21]. The synthesis of the new amine-modified polymers, possessing amphiphilic structures, however, has enabled us to encapsulate the hydrophilic molecule DNA without the degrading effects of shear or ultrasonic forces. The modified solvent displacement method was based on the solubilization of DNA by the polymer, interacting intensively via polyelectrolyte forces in the acetone/water mixture. Final nanoparticle formation in the aqueous medium was a result of the Marangoni effect, which describes the process of droplet formation arising from the rapid diffusion of acetone into the aqueous phase [22]. This results in interface turbulences and small droplet formation. The solvent diffusion process

for nanoparticle formation was described by Quintanar-Guerro et al. [23,24]. In these studies, particle sizes were described to be dependent upon the polymer concentration in the organic phase. This was attributed to the substantial increase of viscosity of the organic phase. Thus, it was found that homologous polymers with increasing molecular weights formed larger nanoparticles, due to higher viscosities [25]. The amine-modified polymers used in our study had very high molecular weights, ranging from M_w 261,600 g/mol to M_w 1199,000 g/mol for P(12)-10 to P(33)-10. PLGA, which was used for comparison had a molecular weight of only M_w 15,200 g/mol, leading to the assumption that the amine-modified polyester particles would exhibit larger hydrodynamic diameters. Remarkably, our new polymers formed smaller particles than PLGA even when nanoparticles were prepared with equal amounts of polymer and DNA (Table 1) [26]. Hence, the amine substitution increased the hydrophilicity of the polymer, which decreased the coalescence rate of the polymer droplets.

The influence of the N/P ratio on the particle size of P(68)-10 nanoparticles revealed that an excess of negative charges of the DNA resulted in large aggregates (Fig.2). This could be explained by the incomplete nanoparticle formation and DNA aggregation. At charge neutrality, sufficient polymer was available to form small nanoparticles. The excess of polymer beyond this resulted in nanoparticles that were mainly regulated in their size by the polymer properties. The ξ-potential values of the P(68)-10 preparations with increasing N/P ratios were expected to increase from the calculations of the nanoparticle stoichiometry. Indeed, nanoparticles with N/P ratios ranging from N/P 1 to N/P 2 exhibited a ξ-potential increase from –49 mV to +51 mV. This complete reversal of the surface charge characteristics demonstrated the ability of the polymer to efficiently encapsulate and compact DNA. Nanoparticles with N/P ratios ranging from 3 to 9 exhibited little change in the ξ-potential. The ξ-potentials of nanoparticles prepared from DEAPA and DEAEA polymers at N/P 5 in Table 1 were, all very similar, demonstrating that a five-fold excess in

amine groups over the phosphate groups resulted in similar surface charges of the nanoparticles independently of the polymer used. P(12)-10 and M(13)-10 nanoparticles had reduced ξ-potentials, possibly resulting from a steric hindrance of amine arrangement in the particle due to higher PLGA grafting per amine group in the polymer.

The polymer mass distribution showed that nearly the total polymer mass could be recovered in the pellet after particle preparation (Table 2). Therefore, the polymers used for the nanoparticles formation were not dissolved in the aqueous medium.

Atomic force micrographs confirmed the PCS data and revealed the nanoparticle structure (Fig.3). The PLGA component seemed to be responsible for the formation of smooth particles. Polymers with a higher proportion of PLGA components compared to the amount of amine groups, for example, M(13)-10 and P(26)-10, had a more defined structure than the E(33)-10 DNA polymer particles. This can be attributed to a reduced interaction of the polymer with DNA or an increased water uptake leading to a collapsed structure interacting more intensively with the silicium support.

The lactate dehydrogenase release assay was performed to investigate the membrane toxicity of the nanoparticles preparations (Fig.4). High cationic surface charges have often been shown to be the cause of cell toxicities [27,28]. No membrane toxicity, defined by a LDH release inferior to 10%, was observed after 3 hours of incubation at 37°C. This preliminary data demonstrated that the nanoparticles preparations in the concentration used are suitable gene delivery agents, exhibiting no membrane toxicity. Further toxicity studies are under investigation.

The release of DNA from nanoparticles at N/P 5 was studied using polymers with increasing degrees of amine substitution (Fig.5). Large amounts of DNA were discovered in the supernatant of the PLGA particles, which implied that the DNA / polymer interaction was low during PLGA particle preparation.

P(12)-10 nanoparticles, despite their cationic charge excess at N/P 5, also allowed free DNA to remain in the supernatant. This can be attributed to a shielding of amine-substituents charges by the PLGA side chains. In this case, the polymer could probably not interact as fully with DNA, compared to higher amine-modified polymers. The DNA encapsulation efficiency for P(33)-10 and P(68)-10 was complete, as no DNA was detected in the supernatant of the preparations. The low DNA release of the P(68)-10 and the absence of release from P(33)-10 was attributed to the strong association of DNA to the polymer and polymer backbone. The protection from DNase increased with the N/P ratio of the P(68)-10 nanoparticles, demonstrating an increase in DNA compactation firstly within the particles and during polymer degradation with the polymer backbone (Fig.6). The DNA in the nanoparticles at a N/P ratio of 9 was protected over 60 minutes.

The association of P(68)-10 nanoparticles with fluorescence labeled DNA was studied to quantify the nanoparticle uptake into fibroblasts (Fig.7). Therefore, the similar incubation conditions as for the transfection experiments were used. The adsorption of the nanoparticles on the cell membranes was reduced by throughoutly washing with low ionic strength buffer, as well as highly concentrated salt solutions. The fluorescence intensity emitted by 10,000 cells was assessed by flow cytometry. Thereby, the intensity increased with the N/P ratio of the nanoparticles. The increase of fluorescence cell association could not be defined as linear compared to the raise of the N/P ratio. This phenomenon was in line with the ξ-potential (Fig.2) and the transfection data (Fig.9b) of P(68)-10 polyester DNA nanoparticles at different N/P ratios. As for the ξ-potentials, this phenomenon could be explained by the formation of nanoparticles that did not have DNA encapsulated within the polymer. Consequently, the uptake of particles carrying DNA and particles without DNA compete for endocytosis. Still, the uptake of the particles with a N/P ratio of 11 is higher that the N/P 9 and N/P ratio of 5.

The nanoparticle uptake into non-phagocytic cells, such as fibroblasts was studied by confocal laser scanning microscopy with fluorescence labeled DNA nanoparticles (Fig.8). Adsorptive endocytic uptake was observed from the 30 min time-point on to the three hour time-point. The DNA and polymer fluorescences thereby mainly remained superimposed. The primary nanoparticle cell membrane interaction took place due to ionic interactions of the cationic nanoparticles with the negatively charged cell glycocalyx. This induced the non-specific endocytosis of the nanoparticles into the non-phagocytic cells. Fluorescence was restricted to distinct areas of the cell for the 30 minutes time-point, leading to the assumption that several particles were internalized in one endosome. After 3 hours of incubation, the particles were dispersed over the cell, arranging themselves in the proximity of the nucleus, which is common for lysosomes. The diffuse green polymer fluorescence in the cytosol of the cell revealed that endosomal escape of the polymer occurred. We hypothesized that the 5-DTAF fluorescein label was mainly bound to PLGA end groups of the polymer. These PLGA end groups of the P(68)-10 polymer are rapidly hydrolyzed, especially in the acidic endosomal environment. Therefore, we explained the diffuse green fluorescence by free fluorescently labeled lactic or glycolic acid in the cytoplasm of the cell. This allows the assumption, that the DNA nanoparticle formulation escaped the endosome. Other groups have explained the PLGA nanoparticle escape from the endosomal pathway by a combination of osmolytic activity localized-destabilization of the membrane that was followed by the extrusion of the nanoparticles into the cytosol [29]. However, we consider that further studies are needed to fully explain the fate of the nanoparticles within the cell. Still, this study demonstrated that DNA particles are intensively taken up by the cells, thus facilitating the gene transfer.

The high transfection efficiencies of the DNA nanoparticles prepared by the modified solvent displacement method demonstrated the potency of this new polymeric system and the bioactivity of the DNA after encapsulation in vitro.

Generally, cationic surface characteristics, and thus, positive ξ-potentials, are required for high transfection efficiencies [30]. This could be observed for P(68)-10 nanoparticles prepared with increasing N/P ratios. The transfection efficiency increased exponentially from N/P 0.5 to N/P 7 (Fig.9b). At the same time, the ξ-potentials of P(68)-10 nanoparticles prepared with increasing N/P ratios exhibited a substantial shift from N/P 0.5 to N/P 5 (Fig.2). Further increase in the cationic polymer excess, however, did not result in considerable changes in ξ-potential or transfection efficiency. This was an indicator that increased DNA / polymer interaction took place in the range of N/P 5 to N/P 7. Higher N/P ratios only resulted in the formation of particles without DNA, which in contrast to water soluble polymers did not show membrane toxicity effects, which could influence the transfection efficiency (Fig.4) [19].

The transfection efficiency of the homologous series of polymer particles was mainly dependent on the degree of amine substitution of the polymer (Fig.9a). Moreover, the polymers with shorter PLGA side chains exhibited higher luciferase expression than polyesters with a 1:20 backbone to PLGA ratio. This correlated with the PLGA degradation of the amine-modified polymers demonstrated by Wittmar et al. [13]. Still, primary experiments in our laboratories revealed that polymer backbone/DNA complexes did not exhibit comparable transfection efficiencies. Therefore, additional factors arising from the polymer PLGA grafting must interfere with the DNA delivery.

While all particles were shown to exhibit similar ξ-potentials (Table 1), their transfection efficiencies greatly increased with the degree of amine substitution. Therefore, the transfection efficiency was not only dependent on the N/P ratio, but it was significantly dependent on the rate of amine modification of the polymeric backbone (Fig.5). It has been demonstrated by others that the charge density and not only the total amount of surface charge mainly influences the transfection efficiency [31]. This effect was further demonstrated comparing the two amine modifications (DEAPA, DEAEA) at N/P 9. DEAPA amine-modified

polyesters were considerably more effective than their DEAEA analogous. It was assumed that the interaction of DNA with the polymer was enhanced by the propyl-spacer (DEAPA) as opposed to the shorter ethyl-spacer (DEAEA), possibly due to reduced accessibility of the positive charge by PLGA shielding. While PEI 25 kDa exhibits a much higher amine density than the P(68)-10 polyester, the enhanced transfection efficiency of P(68)-10 may, in contrast, be attributed to the combination of different effects contributing to an enhanced DNA release from the endosomal compartment. The careful elucidation of the transfection mechanism has yet to be investigated, but different effects could simultaneously interfere during the process [30,32,33]. The efficient amine-modified polymers consisted of dimethlyaminopropylamine substituents, representing tertiary amines, that have been demonstrated to be essential for the endosomal escape of polyplexes by a `proton sponge´ effect [34]. This effect, leading to the osmotic rupture of the endosome and DNA release into the cytosol, could be intensified by the fast polymer degradation, resulting in an increase of the osmotic pressure by acidic degradation products within the endosome, as proposed by Koping-Hoggard [35]. The fast polymer degradation of the amine-modified polymers containing PLGA side chain lengths of 10 monomers, could explained the observed effect, that polymers with shorter PLGA side chains were more efficient in transfecting cells [13,36]. This mechanism would additionally explain the diffuse green fluorescence in the cytoplasm, as well as the reduced transfection efficiency of polymer backbones. However, other mechanisms of endosomal release have eventually to be considered, for example, membrane destabilizing activities, taking into account the low glass transition temperatures and hydrophobic moieties of the polymer, demonstrated by Wittmar et al. [13]. Further, the `hydrogel effect´ proposed by Ishii, describing the swelling of the polymers in the endosome could increase the disruption of endosomes, due to polymer protonation [37]. Therefore, we concluded that the combination of different modules within one biodegradable

polymer, resulting in a fast degrading polymer, ionic interactions with DNA and the formation of water insoluble nanoparticles, provided considerable advantages with regard to the transfection efficiency in vitro.

CONCLUSION

Efficient gene delivery is a prerequisite to reduce the amount of DNA needed for successful DNA vaccination. The novel biodegradable branched polyesters described in this study, composed of an amine-modified PVA backbone with multiple and short hydrophobic PLGA side chains, allowed us to modify the solvent displacement method for DNA nanoparticle preparation. Thereby we were able to encapsulate DNA within biodegradable nanoparticles without the use of high energy sources, as a result of the interaction of DNA by the polymer within the acetone/water solution. The investigation of the polymer series demonstrated that efficient gene delivery, comparable and better than PEI 25 kDa could be achieved in vitro using this nanocarrier system.

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CHAPTER 8

SUMMARY AND OUTLOOK

ZUSAMMENFASSUNG UND AUSBLICK

SUMMARY

In this dissertation different microparticular and nanoparticular DNA carrier systems were developed, with the aim to create an efficient adjuvant system for DNA vaccination.

Their suitability was investigated by physico-chemical parameters, such as particle size, ζ-potential and encapsulation efficiency. Further, the systems were studied in-vitro for DNA stabilization and DNA bioactivity after encapsulation and release, as well as for gene delivery. A promising formulation was finally used as DNA delivery system for in-vivo immunization.

In Chapter 1 we described the basic fundamentals of DNA vaccines, the chances arising from their use, the current research and results indicating the difficulties to reach protective levels of immune responses. Further, vaccine adjuvants were described concentrating on microparticular and nanoparticular systems. These were presented in detail with regard to the preparation techniques and their applications.

In Chapter 3 we investigated modified double emulsion methods and spray drying techniques for DNA microencapsulation. To prevent possible DNA degradation during the encapsulation process, DNA was formulated using several additives. Firstly, DNA was complexed with polyethylenimine (PEI) 25 kDa. We further studied the possibility to encapsulate lyophilized DNA and lyophilized DNA / PEI complexes in the presence of lyoprotectants. The microparticles were formulated using i) a modified double emulsion technique (W/O/W), ii) a solid in oil in water method (S/O/W), iii) a water in oil spray drying technique (W/O) and iv) a solid in oil spray drying technique (S/O). The microparticles were smaller 10 µm for the spray-dried and the W/O/W formulations, thus suitable for phagocytic uptake. DNA release from particles prepared with double-emulsion methods, in contrast to spray drying techniques, resulted in constant DNA release and relatively low initial burst effects. The

complexation with PEI substantially retarded the DNA release for all preparation techniques.

DNA encapsulated in polyester particles is exposed to the acid degradation products of polymer hydrolysis.

In Chapter 4, DNA we adsorbed DNA onto the surface of microparticles. We developed a cationic microparticular system by the incorporation of different amounts of the cationic molecules, PEI or CTAB (Hexadecyltrimethyl-ammonium-bromide), into the polyester matrix. PEI 10% microparticles exhibited the most promising characteristics, such as a small particle size, a high ζ-potential of + 47 mV, a high DNA adsorption efficiency for a theoretical loading of 1% over the physiological pH range. In contrast to the PEI formulations, microparticles containing the detergent CTAB exhibited aggregated particles demonstrated by SEM micrographs, as well as high membrane toxicities and low adsorption efficiencies.

The mechanism of gene delivery was studied by confocal microscopy and revealed diffuse fluorescence of DNA and PEI in the cytoplasm of non-phagocytic L929 fibroblasts. This was attributed to polyplex formation after PEI release from the particle. The efficient gene transfer of RG 502H+PEI 10% microparticles was confirmed by luciferase transfection. Hence, this formulation was chosen for in-vivo DNA immunization against Listeria monocytogenes in mice. The challenge experiments with a lethal dose of the pathogen demonstrated that the formulation had an adjuvant effect.

However, adsorption of DNA onto microparticles by electrostatic interactions can cause instabilities, such as flocculation. In consequence, we encapsulated DNA into nanoparticles to reduce both particle flocculation and DNA degradation.

In Chapter 5 a new polymeric system was designed, consisting of poly (vinyl-alcohol) coupled with diamines, such as diethylaminopropylamine (DEAPA), dimethlyaminopropylamine (DMAPA) or diethylaminoethylamine (DEAEA).

The hydrophilic backbone was further grafted with D,L-lactide and glycolide (50:50) side chains consisting of 10 or 20 monomers. These polymers were characterized by ^{1}H-NMR, gel permeation chromatography-multiple-angle-laser-light-scattering, and differential scanning calorimetry. The amphiphilic properties allowed the formulation of DNA nanoparticles by a modified solvent displacement technique without the use of shear forces. DNA nanoparticles exhibited positive ζ-potentials up to +42 mV. The gene delivery of the nanoparticles was assessed in L929 mouse fibroblasts, which demonstrated high transfection efficiencies, comparable to PEI 25kDa/DNA complexes at a nitrogen to phosphate ratio of 5.

In Chapter 6 we chose one representative polymer, P(26)-10, of the new class of amine-modified polyesters to investigate the influence of several process parameters on the nanoparticle formation. The nanoparticle size was dependent on the volume of the organic solvent as well as on the volume of the aqueous solution. The organic solvent composition further influenced the particle size and the encapsulation efficiency. The variations form parameters of the solvent displacement technique could be explained by polyelectrolyte interactions of the cationic polymer with DNA in the acetone / water mixture. These in consequence influenced the coalescence rate of the polymer.

In Chapter 7 DNA nanoparticles with amine-modified polyesters were further characterized using two classes of polymers (DEAPA /DEAEA) with different amounts of amine modifications. The nanoparticles were prepared at specific nitrogen to phosphate ratios. The nanoparticle ξ-potentials and sizes were dependent on the N/P ratio and highly positive for a N/P ratio higher 3. Atomic force microscopy confirmed the small particle sizes. DNA stability during the encapsulation process and release over nine day was demonstrated by electrophoresis, as well as DNA protection from enzyme degradation in dependence of the N/P ratio.

The amount of cellular uptake of an efficient candidate P(68)-10, DNA nanoparticles was shown to be dependent on the N/P ratio of the formulation by flow cytometry. The mechanism of cellular uptake was followed by confocal microscopy and exhibited endocytotic uptake of the particles. The endosomal escape of the formulation was observed by the covalently bound polymer label in the cytosol and detected by reporter gene expression. The endosomal escape was ascribed to a combination of osmotic effect of the readily degraded PLGA side chains and the polycationic properties of the backbone. The very efficient gene delivery of the P(68)-10 polymer was demonstrated by in-vitro transfection assays in four cell lines compared to PEI / DNA complexes at equal N/P ratios.

OUTLOOK

DNA nanoparticles formulated with amine-modified polymers were demonstrated to be efficient gene delivery systems. In-vivo immunizations with this system are ongoing. DNA nanoparticles of the most promising polymer P(68)-10 are either injected intra-muscularly or applied intra-nasally. Intra-nasal administrations generally were shown to efficiently induce mucosal immune responses.

Further, highly concentrated nanoparticle preparations, especially of the DEAEA-modification, exhibited in-situ aggregation upon injection into buffered medium. Most of the current in-situ forming devices are based on polymer solution in organic solvents. The possibility to inject an aqueous drug - nanoparticle dispersion, which self assembles to a polymeric implant represents a promising possible to develop organic solvent free in-situ implants.

ZUSAMMENFASSUNG

In dieser Arbeit wurden mikropartikuläre und nanopartikuläre DNA-Trägersysteme mit dem Ziel einer adjuvanten Anwendung für DNA-Impfstoffe entwickelt.

Deren Eignung wurde anhand physikalisch-chemischer Parameter, unter anderem ihrer Partikelgrößen und ξ-Potentiale, sowie ihrer Verkapselungs- und Adsorptionseffizienzen bestimmt. Weiterhin wurde in-vitro die schützende Wirkung für DNA vor enzymatischem Abbau, die DNA-Bioaktivität nach deren Freisetzung und die Transfektionseffizienz charakterisiert. Eine optimierte Formulierung wurde schließlich als DNA-Trägersystem für die in-vivo DNA-Immunisierung verwendet.

In Kapitel 1 wurden die Grundlagen der DNA-Immunisierung, deren Möglichkeiten, der aktuelle Stand der Forschung und die Schwierigkeiten der Entwicklung eines effektiven Schutzes durch DNA-Impfstoffe dargestellt. Des weiteren wurden adjuvante Systeme vorgestellt, wobei besonders die Herstellung und Verwendung von bioabbaubaren Mikropartikeln und Nanopartikeln berücksichtigt wurde.

In Kapitel 3 haben wir das Doppelemulsionsverfahren und die Sprühtrocknung zur Herstellung von DNA-Mikropartikeln untersucht. Um den möglichen Abbau der DNA während der Verkapselung zu verhindern wurden unterschiedliche Hilfsstoffe in der Formulierung verwendet. Die DNA wurde in Lösung, als Polyethylenimin (PEI) 25 kDa Komplex, sowie in lyophilisierter Form in Anwesenheit von Lyoprotektoren verkapselt. Folgende Methoden zur Herstellung der DNA Mikropartikel wurden verwendet: (1) ein modifiziertes

Doppelemulsions-verfahren (W/O/W), (2) ein Feststoff in Öl in Wasser Verfahren (S/O/W), (3) die Sprühtrocknung einer Wasser in Öl Dispersion (W/O) und (4) die Sprühtrocknung einer Feststoff in Öl Dispersion (S/O). Die resultierenden Partikel der Sprühtrocknung und des Doppelemulsionsverfahrens waren kleiner als 10 µm und entsprachen daher den Größenanforderungen für eine phagozytotische Aufnahme in Zellen. Die Partikel aus dem Doppelemulsions-verfahren setzten DNA kontinuierlich frei, wohingegen die DNA aus sprühgetrockneten Mikropartikeln primär schlagartig freigesetzt wurde. Durch die Komplexierung mit PEI konnte die DNA-Freisetzung in allen Zubereitungen erheblich verlangsamt werden.

In Polyesterpartikel verkapselte DNA wird durch die sauren Hydrolyseprodukte des Polymers abgebaut.

In Kapitel 4 haben wir daher DNA an Oberflächen von Mikropartikeln adsorbiert. Durch die Integration unterschiedlicher Anteile kationischer Moleküle PEI oder CTAB (Hexadecyl-trimethylammoniumbromid) in Polyestergerüste konnten wir Mikropartikel mit kationischen Oberflächen-eigenschaften entwickeln. Partikel mit 10% PEI zeichneten sich durch besonders positive Eigenschaften, wie zum Beispiel einer geringen Partikelgröße, eines positiven ξ-Potentials von +47 mV und einer besonders guten DNA-Adsorptionseffizienz über den physiologischen pH-Bereich, aus. Diese Formulierung war zudem in der Lage DNA vor enzymatischem Abbau zu schützen. Elektronenmikroskopische Aufnahmen bewiesen, dass CTAB Partikel stark aggregierten und daher erheblich größere Durchmesser als PEI Mikropartikel in der Laser Diffraktometrie aufwiesen. Im Gegensatz zu PEI - Partikeln waren CTAB - Partikel membrantoxisch. Konfokale Aufnahmen mit fluoreszenz-markierten PEI - Partikeln und DNA resultierten in diffuser Fluoreszenz im Zytoplasma von nicht-phagozytischen L929 Fibroblasten. Dies wurde auf das Herauslösen von PEI und der Bildung von Polyplexen zurückgeführt. Der Gen-Transfer der RG 502H+PEI 10% Partikel konnte des

weiteren durch die Transfektion mit dem Reportergen Luciferase bestätigt werden. Daher setzten wir diese Partikel als Trägersystem für die DNA-Immunisierung von Mäusen gegen Listeria monocytogenes ein. Das System bewies dabei einen adjuvanten Effekt auf die DNA-Immunisierung, welches durch die Infektion mit einer letalen Dosis des Erregers untersucht wurde.
Die Adsorption von DNA an kolloidale Systeme, wie kationische Mikropartikel, kann allerdings zu Instabilitäten und Ausflockung führen.

In Kapitel 5 wurde daher die Entwicklung eines neuen Polymersystems für die Verkapselung von DNA beschreiben. Diese Polymere wurden aus Poly-(vinyl-alkohol) und Diamin-Substituenten (Diethylaminopropylamin (DEAPA), Dimethlyaminopropylamin (DMAPA) oder Diethylaminoethylamin (DEAEA) aufgebaut. Diese Polymer-Rückgrate wurden mit Seitenketten aus D,L-Laktid und Glykolid (50:50) aus 10 oder 20 Monomeren gepfropft. Diese neuartigen Polymere wurden über ^{1}H-NMR, Gel-permeation-chromatographie und Differential Scanning Calorimetrie charakterisiert. Die amphiphilen Polymer-eigenschaften ermöglichten es, ein neues Verfahren zu Verkapselung von DNA zu ohne Verwendung von Scherkräften zu entwickeln. Die DNA Nanopartikel zeigten hohe ξ-Potentiale sowie hohe Transfektionseffizienzen in-vitro. Diese waren vergleichbar mit PEI / DNA Komplexe mit einem N/P Verhältnis von 5.

In Kapitel 6 wählten wir ein repräsentatives Polymer dieser neuen Polymerklasse, P(26)-10, um die Prozessparameter des Herstellungsverfahrens der DNA Nanopartikel zu charakterisieren. Die Partikelgröße war abhängig vom Volumen des organischen Lösungsmittels und der wässrigen Phase. Die Zusammensetzung des organischen Lösungsmittels bestimmte durch die Viskosität zusätzlich die Partikelgröße und die DNA Beladungseffizienz. Wir erklärten die Besonderheiten des Systems durch Polyelektrolyt-Wechselwirkungen der DNA mit dem kationischen Polymer in dem Aceton / Wasser Lösungsmittel. Dieses Volumenverhältnis bestimmte daher die Koaleszenzeigenschaften des Polymers mit.

In Kapitel 7 wurden die neuartigen Polymersysteme durch die Formulierung von DNA Nanopartikeln in unterschiedlichen N/P Verhältnissen charakterisiert und in-vitro angewendet. Dabei waren die ξ-Potentiale der Partikel und die Partikelgröße von deren N/P Verhältnis abhängig. Die Raster-Kraft-Mikroskopie konnte die gemessenen Partikelgrößen bestätigen. Zudem wurde DNA durch Verkapselung in Partikel in Abhängigkeit des N/P Verhältnisses gegen enzymatischen Abbau geschützt. Die Freisetzung über 9 Tage zeigte, dass nur Polymere mit hohen Amindichten DNA vollständig verkapseln konnten. Durch Flow Cytometrie mit P(68)-10 DNA Nanopartikeln konnte gezeigt werden, dass die Partikelaufnahme in Zellen vom N/P Verhältnis abhängig ist. Der entsprechende Aufnahmemechanismus wurde mit Hilfe der konfokalen Mikroskopie verfolgt. Die Aufnahme der Partikel in das endo/lysosomale Kompartiment und eine Freisetzung des kovalent gebundenen Polymerlabels in das Cytosol konnte beobachtet werden. Wir nehmen daher eine Freisetzung der Formulierung aus Endosomen durch einen osmotischen Effekt der PLGA Abbauprodukte in Kombination mit polykationischen Eigenschaften des Rückgrates an. Die ausgesprochen gute Transfektionseffizienz eines der Polymere, P(68)-10, wurde in unterschiedlichen N/P Verhältnissen und in vier Zelllinien untersucht und war bei gleichen N/P Verhältnissen vergleichbar mit PEI / DNA Komplexen.

AUSBLICK

Die DNA Nanopartikel des aminmodifizierten Polyesters, P(68)-10 haben sich durch sehr gute Transfektionseffizienzen ausgezeichnet. Daher werden zur Zeit DNA-Immunisierungen durchgeführt. Dazu werden die Nanopartikel-Zubereitungen Mäusen parallel intra-nasal und intra-muskulär verabreicht

werden. Die intra-nasale Immunisierung hat im Allgemeinen den Vorteil, dass durch sie eine verstärkte mukosale Immunantwort entsteht.

Ein zweiter Ansatz verfolgt das Ziel lösungsmittelfreie In-Situ Implantate zu entwickeln. Hochkonzentrierte DNA Nanopartikelsuspensionen, im besonderen die DEAEA – aminmodifizierten Polyester, bilden bei der Injektion in Pufferlösungen Aggregate. Daher könnte die Injektion von wirkstoffhaltigen Nanopartikelsuspensionen in Wasser zur Bildung von bioabbaubaren Depots führen.

APPENDICES

Research Articles

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C.G. Oster, T. Kissel, Encapsulation of DNA into microparticles using modified double emulsion methods and spray drying techniques, in preparation for J. Microencapsulation.

Abstracts

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