DNA gel particles: An overview

M. Carmen Morán[1,2,*], M. Pilar Vinardell[1,2] , M. Rosa Infante[2,3] M. Graça Miguel[4], Björn Lindman[4,5]

[1]*Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Avda. Joan XXIII, 08028 Barcelona, Spain.*
[2] *Interaction of Surfactants with cell membranes. Unit associated with CSIC, Facultat de Farmàcia, Universitat de Barcelona, Avda. Joan XXII, 08028 Barcelona, Spain*
[3] *Departamento de Tecnología de Tensioactivos, IQAC-CSIC, C/Jordi Girona 18-26, 08034 Barcelona, Spain.*
[4]*Department of Chemistry, University of Coimbra, Rua Larga, 3004-535 Coimbra, Portugal.*
[5]*Physical Chemistry 1, University of Lund, P. O. Box 124, 22100 Lund, Sweden.*

*Corresponding author. Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Avda. Joan XXIII, 08028 Barcelona, Spain. Tel:+ 34 93402 45 05; Fax: +34 93402 59 01
*E-mail address:*mcmoranb@ub.edu (M. C. Morán)

Abstract

A general understanding of interactions between DNA and oppositely charged compounds forms the basis for developing novel DNA-based materials, including gel particles. The association strength, which is altered by varying the chemical structure of the cationic cosolute, determines the spatial homogeneity of the gelation process, creating DNA reservoir devices and DNA matrix devices that can be designed to release either single- (ssDNA) or double-stranded (dsDNA). This review covers recent developments on the topic of DNA gel particles formed in water–water emulsion-type interfaces. The degree of DNA entrapment, particle morphology, swelling/dissolution behaviour and DNA release responses are discussed as a function of the nature of the cationic agent used. On the basis of designing DNA gel particles for therapeutic purposes, recent studies on the determination of the surface hydrophobicity, the haemolytic and the cytotoxic assessments of the obtained DNA gel particles have been also reported.

Keywords: DNA gels; DNA-oppositely charged agents interaction; particles; DNA release; haemocompatibility; cytotoxicity

Contents

1. Introduction

The main aim of the gene therapy is to transfer genetic material into the cells to cure diseases through the expression of certain proteins. Despite significant advances in the past couple of decades, gene therapy is still in the clinical trial stage, mainly due to the lack of safe and efficient delivery vehicles for therapeutic nucleic acids. Deoxyribonucleic acid (DNA) is a negatively charged biomacromolecule that is subject to degradation in the bloodstream by endogenous nucleases [1]. Moreover, it is too large to cross the cellular membranes. The most common strategy employed for the "packaging" of DNA is based on electrostatic interaction between the anionic nucleic acid and the positive charges of the synthetic vector which will complex and condense the nucleic acid [2].

The rapidly rising demand for therapeutic grade DNA molecules requires associated improvements in encapsulation and delivery technologies. This includes the formulation of DNA molecules into synthetic delivery systems for enhanced cellular transformation efficiencies. Research works on colloidal delivery systems in genetic therapeutics are based on the molecular level focusing on the interdisciplinary development of pharmaceutical DNA delivery approaches. Colloidal delivery systems modify many physicochemical properties, aiming to protect the DNA from degradation, minimize DNA loss, prevent harmful side effects, enhance DNA targeting, increase drug bioavailability, and stimulate the immune systems [3-5].

Various colloidal systems have been studied for decades improving the delivery of problematic DNA candidates. The most promising systems comprises of ionic complexes formed between DNA and polycationic liposomes [6-9]. Factors hindering the success of the liposomal approach appear to be instability of the complex, toxicity of the cationic lipid, and short half-life of the complexed DNA. Held together by electrostatic interaction, these complexes may dissociate because of the charge screening effect of the polyelectrolytes in the biological fluid. A strongly basic lipid composition can stabilize the complex, but such lipids may be cytotoxic. The fact that the DNA is coated on the outside of the liposome renders the DNA vulnerable to nuclease degradation in transit from site of administration to the nucleus of the target cell.

Biocompatible polymers are widely used in drug delivery and tissue engineering, where they form a variety structures, through either chemical cross-linking or physical association, that range from nanoparticles to macroscopic scaffolds [10-15]. In many applications, it is important to control their degradation rates, which is usually achieved by incorporating hydrolytically or enzymatically cleavable groups into the polymer structure [16-23]. Biodegradable polymer constructs can also be prepared via reversible physical cross-linking, where the degradation rates depend on the strength of electrostatic, hydrophobic, or hydrogen bonding interactions [24-27]. Both of these approaches to tuning the degradation rates, however, have the disadvantages of usually requiring the polymer to be synthetically modified. An alternative approach is to control the degradation rates by using a modular physical cross-linker, the association strength of which will govern the degradation rate of the gel. This can be achieved by exploiting the association between surfactants and polyelectrolytes [28].

Oppositely charged surfactants and polyelectrolytes have a strong tendency to bind to one another. When the surfactant/polyelectrolyte attraction overcomes their solubility in the solvent, associative phase separation occurs [29-34]. This results in the formation of concentrated liquid, gel, or precipitate phases in equilibrium with a dilute liquid [29-37]. The polyelectrolyte chains can assume two types of conformations, either expanded, as in a solution or a hydrogel, or collapsed, such as around a surfactant aggregate as precipitate. Control over the transitions between these states allows exploitation of surfactant and polyelectrolyte mixtures in a wide array of commercial applications, such as drug delivery, cosmetic formulations, and rheological modification. There have initially been two separate reports of associative phase separation in surfactant and polyelectrolyte mixtures (where the polyelectrolyte undergoes a transition from an expanded to a collapsed state) that yields hollow surfactant/polyelectrolyte gel particles, with numerous potential applications in controlled encapsulation and release and separation processes [38-39]. This effect is achieved with a dropwise addition method [40-42] where drops of chitosan solutions are added to an oppositely charged surfactant solution [38-39]. Observations suggest that the gel formation process is diffusion limited, that the gel layer thickness is proportional to the amount of polyelectrolyte in solution, and that the gelled surfactant and polyelectrolyte complex is electroneutral [39].

This behaviour has been exploited to form gel particles by dropwise addition of solutions of different cellulose-based polycations (chitosan, N,N,N-trimethylammonium derivatized hydroxyethyl cellulose (Amerchol JR-400)) [38-39, 43-44] to anionic (sodium dodecyl sulfate (SDS), sodium perfluorooctanoate (FC7)) and catanionic (cetyltrimethylammonium bromide (C16TAB)/sodium perfluorooctanoate (FC7)) surfactant solutions [45]. This approach has been extended to encapsulate an aromatic oil in surfactant–polyelectrolyte gel particles. Its release into either aqueous or organic phases has been studied [46].

A general understanding of the interactions between DNA and oppositely charged agents, and in particular the phase behaviour, has given us a basis for developing novel DNA-based materials, including gels, membranes and gel particles [47]. Concerning DNA gel particles, we have recently prepared novel DNA gel particles based on associative phase separation and interfacial diffusion. By mixing solutions of DNA (either single- (ssDNA) or double-stranded (dsDNA)) with solutions of different cationic agents, such as surfactants, proteins and polysacharides, the possibility of formation of DNA gel particles without adding any kind of cross-linker or organic solvent has been confirmed [48-51]. The association strength, which is tuned by varying the chemical structure of the cationic agent, allows a control of the spatial homogeneity of the gelation process, producing either a homogeneous DNA matrix or different DNA reservoir devices.

Cationic surfactants have offered a particularly efficient control of the properties of these DNA-based particles [47-57]. We have initially exploited to form DNA gel particles at water–water emulsion-type interfaces by mixing DNA (either single- (ssDNA) or double-stranded (dsDNA)) with quaternary ammonium surfactants. The formation of a physical network in which surfactant micelles form polyanionic−multicationic electrostatic complexes as cross-link points seems to play an important role in the stabilization of DNA particles. Changes in the hydrophobic moiety of the surfactants affect their interaction with DNA [53-54]. The interactions of surfactants with DNA can also be tuned efficiently by controlling the head-group structure. These include surfactants with the cationic functionality based on an amino acid [57]. Surfactant molecules from renewable raw materials that mimic natural lipoamino acids are one of the preferred choices for food, pharmaceutical and cosmetic applications. Given their natural and simple structure, they show low toxicity and quick biodegradation [58]. Novel DNA gel particles, in which surfactants with the cationic

functionality is based on the amino acid arginine, can interact with nucleic acids to form biocompatible devices for the controlled encapsulation and release of DNA [55].

Little is known about the influence of the respective counter-ions on surfactant-DNA interaction. In general, oppositely charged macro-ions in solution attract each other, tending to form a bound complex. When separated, each macro-ion is surrounded by a diffuse layer of spatially confined counter-ions. Upon approach, the fixed macro-ion charges partially (sometimes fully) neutralize each other, allowing the release of mobile counter-ions into the bulk solution, thereby increasing their translational entropy. This scenario suggests that macro-ion association in solution is to a large extent an entropically driven process [59]. The actual contribution of counter-ion entropy to free energy association depends on the detailed geometries and charge distributions of the separated and bound macro-ions [59-61]. In this context, the effect of different counter-ions on the formation and properties of DNA gel particles, by mixing DNA with the single-chain surfactant dodecyltrimethylammonium (DTA) has been recently investigated [54]. In particular, we employed, as counter-ions of this surfactant, anions of the two extremes in the Hofmeister series (hydrogen sulfate and trifluoromethane sulfonate) and two halides (chloride and bromide). The obtained results indicate that the degree of counter-ion dissociation from the surfactant micelles and the polar/hydrophobic character of the counter-ion are important parameters in the final properties of the particles.

Most of the studies about the interaction between polyelectrolyte gels and oppositely charged surfactant systems have been carried out using single chain surfactants. However, most transfection lipids are not micellar [62]. Consequently, the extension of micelle to vesicle-forming double tail surfactants seems to be crucial. The formation of DNA gel particles, mixing DNA with the double chain surfactant didodecyldimethylammonium bromide (DDAB) has been studied [56]. DDAB–DNA gel particles have been shown to form ordered multi-vesicular assemblies which resemble the architecture of biological tissues. The characteristic sizes and shapes of the resulting structures strongly depend on the secondary structure of the DNA. Some features about the DNA–vesicle interaction involved in these particles have been deduced.

The development of biodegradable, biocompatible DNA gel particles has been achieved using natural proteins or mixtures of proteins [52, 63-64] Lysozyme is one of

the main proteins in hen egg white and it has an ability to cause lysis of bacterial cells [65]. Lysozyme is a globular protein that has a net charge of +9 at neutral pH, and was initially used for the formation of DNA gel particles [52]. Protamines are positively charged (overall charge +21), arginine rich proteins that bind to DNA in a non-specific manner via electrostatic interactions. In addition, protamine sulfate has been shown to condense DNA [66-68] and to deliver plasmid DNA into eukaryotic cells [69]. This property, in addition to its longtime use in pharmaceutical formulations, makes protamine a promising candidate for gene delivery. Using mixtures of both proteins as DNA carriers, we have obtained systems totally based on biocompatible components, with a large degree of control over the release profile [63.

Chitosan is a natural, biodegradable polysaccharide derived from chitin and its low toxicity has been well established. Chitosan has been proposed as an attractive gene carrier because of its high density of positive charges and its low toxicity to cells [70]. A number of *in vitro* studies showed that chitosan is a suitable material for efficient, non-viral gene and DNA vaccine delivery [71-73]. Novel chitosan-DNA gel particles have been prepared based on associative phase separation and interfacial diffusion using mixtures of DNA and chitosan of different molecular weight [74-75]. In particular it was found that the chitosan molecular weight is a good controlling parameter in the final properties of these DNA gel particles [74]. More recently, mixtures of chitosan with proteins were used as intrinsic biocompatible carriers to form DNA gel particles. Controlling the magnitude of the DNA release and achieving controlled release systems were accomplished using these ternary systems [75].

Table 1 summarizes the characteristics of the different DNA gel particle systems. These systems can represent a ‘‘bridge’’ for potential applications in the controlled encapsulation and release of ssDNA and dsDNA. The goal of this Review is to explore current research in DNA gel particles prepared by associative phase separation and interfacial diffusion. In the following sections, we describe the influence of the nature of the cationic agent, i.e. surfactant, protein or polysaccharide, on the degree of DNA entrapment, particle morphology, swelling/dissolution behaviour, and DNA release responses. Recent studies on the determination of the surface hydrophobicity, the haemolytic and the cytotoxic characterization of the obtained DNA gel particles have been also discussed.

[Table 1 here]

2. Particle preparation

As described above, interactions between oppositely charged surfactants and polyelectrolytes in aqueous solutions can lead to associative phase separation, where the concentrated phase assumes the form of a viscous liquid, gel, liquid crystal or precipitate. This behavior has been initially exploited to form DNA gel particles at water–water emulsion-type interfaces by mixing DNA (either single- (ssDNA) or double-stranded (dsDNA)) with the cationic surfactant cetyltrimetrylammonium bromide (CTAB) [52] .At high polyelectrolyte concentrations, droplets from DNA solutions instantaneously gelled into discrete particles upon contact with the corresponding surfactant solutions, as is depicted in Fig. 1a. The size of the resulting particle reflects the size of the parent drop and varies between 1 and 2 mm (Fig. 1b). Similar results were obtained when the cationic surfactant was substituted for a protein or polysaccharide molecule. All DNA gel particles were formulated using the method of simple complexation between molar concentrations of negative charge of the phosphate groups of DNA, and the positive charge of the cationic compound. Particles were prepared at a ratio between DNA and the different cationic compounds equal to 1, R= [DNA]/[C^+]. In all cases, the DNA concentration was set to 60 mM. The choice of DNA concentration reflects the fact that it produces high viscosity solutions, which makes it a convenient system for the preparation of stable DNA gel particles [52].

[Fig. 1 here]

Although the molecular details of the mechanism by which cationic carriers mediate DNA delivery are still poorly understood, current evidence supports the hypothesis that theDNA complexes enter cells by means of endocytosis. Often, the particle size ranges from 100 nm to higher than 1 mm, and, evidently, the efficiency of cellular uptake and subsequent intracellular processing, a prerequisite for effective cellular transfection, may well depend on particle size [76]. To address the problem of the size of the obtained DNA gel particles, a simple and novel method was used for the preparation of nano-/micro-sized DNA gel particles by nebulisation of a solution of DNA (single- or double-stranded) into an oppositely charged surfactant or protein solution [77]. Particles were prepared at a ratio between DNA and cationic agent equal to 1. In all cases, [DNA] was equal to 5 mM. Higher concentrations of DNA produce high viscosity solutions, which make them inconvenient systems for the nebulisation process. It was found that

the size of the initial DNAdroplets and the cationic agent are the main controlling parameters for the particle size (Table 2).

3. Physicochemical characterization

3.1. Degree of DNA entrapment

It is of major interest to characterize the degree of DNA entrapment on the DNA gel particles. The degree of DNA entrapment can be expressed as a function of the loading efficiency (LE) and loading capacity (LC) values. LE measures the amount of DNA that is included in the particles with respect to the total DNA, during particle formation. LC measures the amount of DNA entrapped inside the particles as a function of their weight. Characteristics of these DNA gel particles, which are formed using surfactants, proteins, and polysaccharides as cationic compounds, are summarized in Fig. 2. All values were measured in triplicate and are given as average and standard deviation.

Except in the case of the double-tail surfactant DDAB, the LE values were always higher than 99%, which confirms the effectiveness of DNA entrapment in cationic solutions derived from the assayed surfactants, proteins and polysaccharides (Fig. 2a). However, the entrapped DNA, as a function of the weight of the particles (LC values), depends on the cationic compound used (Fig 2b). The LC values obtained for surfactant-DNA gel particles depends on the hydrophilic and hydrophobic contributions. For the same type of polar head, trimethylammonium bromide type (CTAB and DTAB structures), the hydrophobic contribution did not have a strong influence on the observed LC value [53-54]. Identical LC values were obtained when DNA gel particles were prepared with surfactants that only differ in counter-ion structure (DTAB, DTAC, DTATf) [54]. However, for the same hydrophobic chain length, 12 carbon atoms in the hydrophobic chain (DTAB, ALA and LAM structures), there is a clear effect of the number of charges on the polar head. Whereas DTAB and LAM showed one positive charge on the polar head, the ALA structure showed two positive charges. Accordingly, higher the number of charges, higher the LC values [10]. When single and double-tail surfactants were compared (DTAB and DDAB structures), we can conclude that higher number of hydrophobic chains on the surfactant structure contributes negatively to the LC values [56].

In the case of protein-DNA gel particles, the lowest LC value (0.7%) was obtained in particles formed with pure lysozyme LS. Interestingly, highest LC value were obtained for particles containing the pure protein PS or mixed systems containing the smallest amount of protamine sulfate PS in the mixed protein systems [63]. These differences could be attributed to differences in the binding characteristics of these two proteins, with different total charge and linear charge density: LS is a globular protein that has a net charge of + 9 at neutral pH, whereas PS is a highly positively charged linear protein with an overall charge of +21. The formation of the chitosan- DNA gel particles was studied using mixtures of DNA and chitosan of different molecular weight ((Low MW chitosans of ca. 50 kDa and 150 kDa, and medium MW chitosan (400 kDa) [74] .The molecular weight of the polysaccharide structure did not have a clear effect on the LC values.

An indication of the structural characteristics of these DNA gel particles can be deduced from the amount of DNA released, when particles' breakup is mechanically promoted. The percentages of DNA complexed were calculated and are summarized in Fig. 2c. Complexed DNA is related to the amounts of DNA in the supernatant solutions and the skins derived from the particles, after particles were magnetically stirred overnight. In the case of surfactant-DNA gel particles, the percentages of complexed DNA suggest that, with surfactant structures with twelve carbon atoms in the hydrophobic chain (DTAB, DTAC, DTATf, ALA, LAM), most of the DNA is complexed during the particle formation process [54-55]. More limited complexation has been obtained by increasing, either the alkyl chain length at to sixteen carbon atoms (CTAB), or the number of alkyl chains in the molecule from one to two (DDAB) [53, 56]. In the case of the protein-DNA gel particles, the amount of complexed DNA increases progressively in the presence of the protein PS [63] as a consequence of the binding characteristics of this protein. The amount of complexed DNA in the case of chitosan-DNA gel particles seems to decrease when the molecular weight of the polysaccharide is increased [74]. A low molecular weight of chitosan promotes the formation of particles in which a higher percentage of DNA is complexed. The continuous diffusion of chitosan throughout the particles promotes the increase of the shell portion with time.

This distribution could be correlated with differences in the gelation process during particle formation. Homogeneous gelation can lead to homogeneous structures (solid

particles), whereas a more inhomogeneous gelation process forms core-shell structures [43]. In the present study, the model distribution of DNA in the particles was supported by visual inspection, since translucent core-shell particles and opaque condensed particles were found (Fig. 2d).

[Fig. 2 here]

3.2. Morphological characterization of the DNA gel particles

The secondary structure of the DNA molecules in the gels was studied by fluorescence microscopy (FM) using acridine orange staining. Acridine orange (AO) has been used to label nucleic acids in solution and in intact cells [78-81]. In the case of AO-dsDNA, the fluorescence emission shows a maximum around 530 nm, in the green spectra. The association with ssDNA shows a maximum around 640 nm, in the red spectra.

Based on the observation of green or red emission, AO was used to differentiate between native, double-stranded (dsDNA), and denatured, single-stranded (ssDNA), in DNA gel particles. Fig. 3a shows fluorescence micrographs of individual particles of the surfactant-dsDNA systems. FM studies have revealed that the formation of particles with double-stranded DNA is carried out with conservation of the secondary structure of the DNA. However, in the case of particles formed with denatured DNA, green emission is also observed, except in the case of CTAB-ssDNA gel particles. The absence of red emission in the particles containing denatured DNA suggests that the accessibility of free DNA to the dye is hindered. This observation is consistent with our data on DNA distribution (Fig. 3b). The percentage of DNA released was less than 0.1%, which confirms the total complexation of the DNA. However, when CTAB was used, the amount of ssDNA released reached 20%, making its detection possible in fluorescence microscopy studies.

Scanning electron microscopy imaging was carried out to establish possible differences in the morphologies between the different particles. Fig. 4 shows representative images of CTAB and DTAB surfactants. Clear similarities were found in the outer surface morphology between these four formulations. However, the surface of the inner structure revealed a different structure. Large pores and channel-like structures were found in the inner surface of particles formed with CTAB. However, the structure

of the particles formed with DTAB revealed a more compact structure. The structures obtained seem to confirm the degree of complexation between these two surfactants and DNA (see Fig. 3b), which increases the shell section of the obtained particles.

[Fig. 3 here]

[Fig. 4 here]

Similar experiments were carried out on particles formed with the double-tail surfactant DDAB [54]. The examination of the DDAB-DNA gel particles with the DNA-selective dye AO revealed the formation of spherical domains on the surface of these DDAB-DNA gel particles (Fig. 5a). The nature of these domains was studied using the hydrophobic dye Nile Red (NR) (Fig. 5b) [82]. The fluorescence emission of NR, in the presence of DDAB-DNA gel particles, was nearly identical to that recorded for DDAB vesicles (results not shown). This indicates that the observed spherical domains on the surface of the DDAB-DNA gel particles are composed of hydrophobic layers of the surfactant. SEM experiments also confirm the presence of these spherical domains (Fig. 5c).

Differences in the reorganization of DNA were found as a function of the secondary structure using both FM and SEM. In the case of particles formed with native DNA, the observed vesicular domains seem to have grown by fusion of several vesicles, adopting a near-spherical shape. However, the greater thickness of the vesicular domains found in the DDAB–ssDNA particles suggests that the reorganization of DDAB vesicles in the presence of denatured DNA takes place with the subsequent formation of multilamellar complexes. Although these DDAB–DNA particles were prepared at the same DNA/DDAB ratio, the results indicate that differences in local DNA concentration or some kind of inaccessibility of one of the components can be significant. FM images at higher magnification also support these differences.

[Fig. 5 here]

3.3. Swelling/dissolution behaviour and kinetics of DNA release

Gels are considered to have great potential as drug reservoirs. Loaded drugs can be released by diffusion from the gels or by gel erosion. When the DNA gel particles are

inserted into a certain medium, different responses occur: swelling or deswelling, dissolution, and release of DNA.

The swelling protocol begins when the initial weight (W_i) of the particles prepared under each condition was measured immediately after were prepared, separated by filtration and washed with pH 7.4 PBS to remove excess of salt. Particles (around 100 mg) were exposed to the initial PBS buffer at an agitation rate of 30 rpm and at room temperature, using a shaking platform. Additionally, the wet particles were measured at each time point (W_t) immediately upon removal of the release solution. Then, fresh solution was added in order to maintain a clean environment. The swelling ratio of the particles at each time point was calculated accordingly from the following equation:

Relative weight (RW) = W_t / W_i

This value reflects the change in weight of the particles at each time point with respect to the initial weight of the gel. Complete degradation of a particle sample was noted when the presence of the particles or fragments of them were no longer visually apparent.

Simultaneously to the studies of swelling/dissolution behaviour, DNA release studies were carried out. The release solution was completely removed from the samples and completely replaced with fresh solution at periodic time points. The amount of DNA in the release solutions was quantified by measuring the absorbance at 260 nm. The cumulative DNA release was normalized with respect to the initial weight particle and expressed as percentage.

In the case of surfactant-DNA gel particles, the extension of the swelling process depends on the surfactant structure (Fig 6a). For the same hydrophilic contribution (CTAB and DTAB), the decrease in the number of carbon atoms from sixteen to twelve in the hydrophobic chain contributes negatively to the swelling extent. So, when CTAB-DNA gel particles were placed in pH 7.6 10 mM Tris HCl buffer, water was taken up from the medium and swelling could be observed. The swelling continued during the entire time interval studied (1,200 h) [53]. However, DTAB–DNA particles showed an initial swelling and then dissolved completely after 48 h [54].

Generally, the DNA release pattern resembles that observed in the swelling/dissolution profiles. Thus, CTAB-DNA particles placed in pH 7.6 10 mM Tris

HCl buffer showed no initial burst release (Fig.7a). After 1,200 h, 69 % of DNA was released from the particles [8]. Nevertheless, DTAB–DNA particles exhibited fast release behaviour by a dissolution mechanism. The corresponding half-life of DTAB–dsDNA is 4. After 24 h, more than 97% of the bound DNA was released [54].

For the same hydrophobic contribution (ALA and LAM derivatives), the swelling/dissolution behaviour can be modulated by modification of the type and number of positive charges on the polar head [55]. Particles containing ALA exhibited the largest (relative weight ratio, RW 2.5) and the longest (more than 1,300 h) swelling process. Particles containing LAM swelled (RW 2.0 using the maximum points as estimate) for up to 200 h, and then started to shrink. More stable particles were obtained for ALA than for LAM, probably due to its double charge. Thus, LAM-DNA particles exhibited faster release than ALA-DNA particles (Fig. 7a). Complete release from LAM-dsDNA particles occurred after 400 h. When the formulation contained ALA, the DNA release was slower. Complete DNA release was only achieved after 1800 h. The results suggest that, for the same hydrophobic contribution, the stability of the gel particles is additionally given by the electrostatic interaction between DNA and the oppositely charged surfactant.

Furthermore, the increase in the number of alkyl chains from one to two (DDAB) contributes positively to the stabilization of the particles [56]. DDAB-DNA particles absorbed an amount of water that was twice the initial mass (relative weight, RW, of 2, using the maximum points as an estimate). These particles had returned to the original particle weight by the end of the experiment (1,500 h). In the case of DNA gel particles formed using the double-tail surfactant DDAB, an initial burst release was observed (Fig 7a). The amount of DNA released in the first 24 h was 44% for DDAB–dsDNA particles. The presence of this burst suggests that some DNA is not encapsulated, or DNA is bound weakly on the surface of the particles. From 24 to 600 h, a plateau was observed in the cumulative DNA release. After that, particles placed in the buffer solution showed a change in release kinetics. A linear cumulative release was observed until the end of the experiment (1,500 h). The amount of DNA released from DDAB–dsDNA was 63%.

Concerning protein-DNA gel particles, LS-dsDNA gel particles lost weight rapidly and extensively (Fig. 6b) [63]. In the case of PS-dsDNA particles, the largest relative

weight ratio was observed (RW>5). For the high LS/PS ratio, particles absorbed a water amount of 2-3 times the initial mass (relative weight ratio, RW, of 2-3) during the swelling process. With a decrease in the LS/PS ratio, more moderate absorption of water was observed (RW=1-2). When the particles contained PS, there was a common trend in the swelling profiles, in which initial swelling was visible before the particle started to dissolve. The initial period in the swollen state, before dissolution takes place, was independent of the PS content and lasted approximately 100 h (using the maximum of the first peak as an estimate). Then, a short period of stabilization was observed after a new, more limited, maximum appeared (located around 400 h). Thereafter, the RW value became approximately constant, with two exceptions. For PS-dsDNA particles, RW increased with time, while for the LS-PS15 system, nonmonotonic behaviour was observed.

LS-dsDNA particles exhibited fast burst release behaviour by a dissolution mechanism (Fig. 7b). After 24 h, 84% of the bound DNA was released. When the formulation contained PS, the initial burst release was absent. The percentage of DNA released in the dissolution media, after 24 h, varied from 0.4 to 1.0% for protein mixed systems. The absence of a burst effect suggests that minimal amounts of unencapsulated DNA are present on the surface of the particles after their formation. For particles containing both proteins, the profiles showed slower DNA release than in the pure systems. The release rates remained almost constant in the case of particles formed at a high LS/PS ratio (LS-PS15 and LS-PS30). However, with a decrease in the LS/PS ratio, a sudden acceleration of the release was observed after ≈400 h. We can assume that complete hydration of the core in our particles could occur after 400 h, taking into account the presence of the maximum RW values in the swelling-dissolution experiments (Fig. 6b). This matrix swelling behaviour could determine the change in the rate of DNA release, which became dependent on the LS/PS ratio. In addition, the final release percentage was largely dependent on the LS/PS ratio. As indicated by the arrow in Fig. 6, the formulations with the lowest PS content released only a small percentage of the DNA present in the particles (<20%), but this percentage increased with PS content to attain ca. 80% for the LS-PS85 formulation.

The determination of the kinetics of swelling and dissolution behaviour demonstrated that chitosan-DNA gel particles lost weight rapidly and extensively (Fig. 6c). The molecular weight of chitosan has a significant effect on the encapsulation of

DNA and on the *in vitro* release properties. The release of DNA from the different particles is illustrated in Fig. 7c. Generally, DNA release rates in the initial period were high in all cases. In the first 24 hours, 57%, 71%, and 74% of DNA were released from the particles containing low MW chitosans of ca. 50 kDa and 150 kDa, and medium MW chitosan (400 kDa), respectively [74].

[Fig. 6 here]

[Fig. 7 here]

3.4. Surface hydrophobicity evaluation of DNA gel particles

Surface properties of carriers determine their physicochemical characteristics and fate in blood circulation [83]. Information about surface properties is of high relevance for carriers, especially for those intended for parenteral delivery. Hydrophobicity of the particle surface governs the adsorption of plasma proteins [84] and subsequently the rate of clearance from systemic circulation. These parameters also influence the overall bioavailability of the drug/ gene delivered by the tested nanocarrier.

The hydrophobicity of a particle surface has been shown to influence not only the amounts of protein bound to the particle, but also the identities of the bound proteins [85]. The number of proteins bound to the particles increased with increasing hydrophobicity of the particle surface, as well as with increasing size. Generally, hydrophobic particles are opsonized more quickly than hydrophilic particles, due to the enhanced absorbability of plasma proteins onto the surface of hydrophobic particles [86-89]. The roles of particle size, surface curvature, and particle surface area in protein binding have also been investigated. Investigators have shown that, the amount of bound protein varied with size and surface curvature. However, the protein pattern was the same for all sizes considered [90]. Though particle composition (base material type, shape, and size) clearly influences protein binding, the surface properties (charge and hydrophobicity) are likely to be more important.

There are only a few ways to measure or compare the hydrophobicity of surfaces in disperse systems. Contact-angle measurements cannot be applied to the hydrated colloidal particle in its original dispersion medium. Polymer particles need to be dissolved in an organic liquid to cast the polymer film for the contact-angle

measurements. The properties of the dry polymer film certainly differ from the properties of a strongly curved surface; therefore, it is insufficient to determine only the contact angle on macroscopic surface with conventional measurements. In the last few years, the determination of the so-called surface hydrophobicity of colloidal particles and biopolymers has been improved [91]. In this set of experiments, the adsorption of hydrophobic yet soluble organic dyes onto different polymer lattices has been investigated. The measurements have been conducted using a UV-VIS spectrophometer. The advantage in measuring in the visible spectrum is that impurities (surfactants, monomers) released by the colloidal particles over time do not disturb the spectrum of the dyes. Rose Bengal (RB) partitioning method was developed as an easy and quick method to estimate the surface properties of uncoated polymer nanoparticles [92]. Rose Bengal is a xanthenes dye used as photosensitizer, fluorescent label and as adsorption marker [91, 93].

The first approach on the determination of the superficial hydrophobicity of DNA gel particles have been carried out on DNA gel particles formed by mixing DNA with chitosan lactate (CL) (binary systems) or CL in combination with the protein protamine sulfate (PS) (ternary systems) [75]. For this purpose, the RB adsorption on the CL-DNA gel particles prepared at different ratio R values was studied. The quantity of free RB in the solution was determined by interpolation from a calibration curve. The concentration of RB bound to the particle surface was calculated as the difference between the total concentrations of RB used in the assay and free RB. The obtained data were transformed to the adsorption isotherms (Γ), which is correspond to the RB bound as a function of the particle weight , in respect to the RB at the equilibrium (free RB). Fig. 8a shows the evolution of the adsorption isotherms as a function of the ratio R values. All DNA gel particles were formulated using the method of simple complexation between molar concentrations of negative charge of the phosphate groups of DNA, and the positive charge of the cationic compound. Particles were formed at specific R ratio, where R=[DNA]/[C^+]. In the case of binary systems, R values varied between and 39.6 and 1, where [DNA] and [C^{+}] are expressed in % (w/v). In the case of ternary systems, particles were prepared at a ratio R equal to 1, where [C^+] is the concentration of the corresponding cationic system, expressed in % (w/v). The composition of the mixed systems was varied between 15 and 85% PS. In all cases [DNA] was equal to 2 % (w/v).

Due to the relative fragility of the CL-DNA particles prepared at R=7.92, there were not enough individual particles to carry out these experiments. In general, it can be deduced that the maxima of RB adsorption increases when the R value is decreased. The shape of the isotherms is not sophisticated at all, showing the decay of the adsorption at higher equilibrium concentrations. This decay is mainly observed for the systems prepared at lower R values, for which higher concentration of CL on the particles is expected. The observed decay on the adsorption isotherms can be correlated with the aggregate formation of the dye as have been observed in the case of RB with cationic surfactant molecules [94]. Similar experiments were carried out with the CL/PS-DNA gel particles prepared at different composition (Fig. 8b). Due that the particles formed with pure PS exhibit a strong tendency to aggregate; no RB adsorption experiments were carried out with this pure protein system. In general, it can be deduced that the RB adsorption increases when the percentage of PS in the composition are increased. The decay of the RB adsorption at higher equilibrium concentrations becomes less important when the percentage of PS in the composition is increased.

Taking into account the Γ_{max} value, the relative hydrophobicity of these DNA gel particles has been established (Fig. 8c) It is expected that the higher the Γ_{max} value, the higher is the relative hydrophobicity. The obtained results suggest that the surface hydrophobicity of the particles using binary systems is a function of the R values, increasing when the concentration of CL is increased. On the ternary systems, the surface hydrophobicity of the particles is a function of the composition, increasing when the concentration of PS on the mixtures is increased.

Due that the obtained results suppose the first approach on the determination of the superficial hydrophobicity of DNA gel particles, in order to grade their relative hydrophobicity, these values have been compared with other described in the literature, in which the Γ_{max} values of several polymer lattices have been shown [91]. Particles derived from polystyrene, which presents a strong hydrophobic character showed Γ_{max} values close to 0.072 mmol g^{-1}. On the other hand, Γ_{max} values for poly(methyl methacrylate) particles, that present a hydrophilic character, were found to be 0.017 and 0.037 mmol g^{-1}, depending on the ionic character. Considering those values, it can be deduced that particles obtained using both ternary and binary systems showed a strong hydrophilic character with Γ_{max} values ranged between $5x\ 10^{-4}$ - $2.84x\ 10^{-3}$ mmol g^{-1}, and $9.1x\ 10^{-5}$ - $5x\ 10^{-4}$ mmol g^{-1}, respectively. The acute hydrophilic character of these

DNA gel particles may govern the posterior adsorption of plasma proteins and influence the overall bioavailability of the system.

[Fig. 8 here]

4. Determination of *in vitro* biocompatibility

At a consensus conference of the European Society for Biomaterials in 1986, the word "biocompatibility" was defined as "the ability of a material to perform with an appropriate host response in a specific application". With the rapid development of biomaterials, the scope of "biocompatibility" has been widely broadened. Herein, we use biocompatibility to include the deleterious effects caused by the DNA gel particles, covering the *in vitro* haemolytic and cytotoxic assessments.

4.1. Haemolysis

An important feature in the development of particulate systems for parenteral administration is to determine their ability to cause haemolysis by interaction with the cell membrane. The potential uses of colloidal self-assemblies as drug delivery systems make haemolysis evaluation very important. To this end, we examined this interaction by using erythrocytes as a model biological membrane system, since erythrocytes have been used as a suitable model for studying the interaction of amphiphiles with biological membranes [95-97]. Most *in vitro* studies of surfactant-induced haemolysis evaluate the percentage of haemolysis by spectrophotometry, to detect plasma-free haemoglobin derivatives after incubating surfactant solutions with blood and then separating undamaged cells by centrifugation. However, in the case of particles, the interpretation of the results of these studies is complicated, due to the variability of experimental approaches and a lack of universally accepted criteria for determining test-result validity.

The first approach on the determination of the haemolytic response of DNA gel particles have been carried out on DNA gel particles formed by mixing DNA (either single- (ssDNA) or double-stranded (dsDNA)) with different single-chain surfactants whose structure differs only in the corresponding counter-ion [54]. First of all, the haemolytic potency of the different components was determined separately. The dependence of haemolysis on the concentration of the surfactant is shown in Fig. 9a. In this experiment, haemolysis was determined at a fixed time, after 10 min incubation in

the presence of various surfactant concentrations. Haemolysis varied with the surfactant concentration in a sigmoidal manner. At concentrations below 300 μg mL^{-1}, for DTAB and DTATf surfactants, the percentage of haemolysis was not significant (below <5 %), which can be regarded as a non-toxic effect level. However, it increased sharply between 400 and 600 (or 700 μg mL^{-1}, depending on the surfactant structure) to reach essentially 100% haemolysis at that concentration. The concentrations assayed were well below 4,000-4,500 μgmL^{-1}, which corresponds to the CMC (Critical Micelle Concentration) value of the surfactants, as previously determined (5. 0 mM DTATf [98], 15.0 mM DTAB [99], 20.0 mM DTAC [100]). The sigmoidal pattern of this DTA–induced haemolysis is indicative of a complex process in which sufficient surfactant needs to accumulate in the target membrane to induce the osmotic lysis of erythrocytes. The HC_{50} values for the different surfactant structures are 443, 468 and 510 μg mL^{-1} for DTAC, DTATf and DTAB, respectively. The haemolytic potency of the DNA was also determined. As expected, DNA showed no haemolytic activity.

[Fig. 9 here]

One drawback of these surfactant-DNA gel particles, in toxicological terms, is the need for a cationic surfactant, which may cause some cellular damage. Our results indicate, however, that the effect of the surfactant can be modulated when administered in the DNA gel particles, unlike what happens in aqueous solution. This modulation is due to the strong interaction between the surfactant and the biopolymer, which leads to a very slow release of the surfactant from the vehicle. Accordingly, although the HC_{50} values for these three surfactants are very close in aqueous solution, strong differences were found when the haemolysis kinetics of the corresponding surfactant-DNA gel particles was determined, as represented in Fig. 9b and Fig. 9c. As the haemolytic character of these surfactants in solution is almost identical, the differences found in the haemolysis responses induced by the different surfactants in the DNA particles can only be related with the capacity to form weaker or stronger surfactant-DNA complexes. It is then expected that for a higher degree of complexation, less surfactant, which could interact with the erythrocytes membrane, would be released in solution.

This trend in surfactant-DNA interaction reflects both the release of haemoglobin (degree of haemolysis) and the release of DNA into the media, as a consequence of different dissolution kinetics of the polyelectrolyte-surfactant complexes. Under the experimental conditions in which the haemolysis studies were carried out, dsDNA-

surfactant particles were fully dissolved by the end of the experiments. However, ssDNA-surfactant particles remained visible in the dispersion. Here, for the first time, both parameters were determined simultaneously, giving us information about the effectiveness of the two release processes. Fig. 10 shows the relative kinetics of DNA and haemoglobin release.

[Fig. 10 here]

The amount of DNA that is released and the haemolytic response are strongly dependent on both the structure of the counter-ion in the surfactant and the secondary structure of the DNA. In the case of particles prepared with native DNA, the amount of dsDNA that is released at the end of the experiment (180 min) reaches 100 μg mL^{-1} (Fig. 10a). However, with particles prepared with denatured DNA, only 10% of this amount is released into the media (Fig. 10b). This behaviour, which can be correlated with the degree of complexation, is higher in the case of ssDNA, thus decreasing the amount of non-complexed DNA that could be detected in solution. These differences are also supported by visual inspection: surfactant-dsDNA particles are completely dissolved at the end of the experiment, whereas surfactant-ssDNA particles are still present after 180 min.

At this point, it is possible to establish which of these systems is the most haemocompatible. For this, the haemolysis values for a defined amount of released DNA are compared. In the case of the surfactant-dsDNA particles, for a concentration of dsDNA equal to 100 μg mL^{-1}, the degree of haemolysis is 30%, 60% and 80%, when DTATf, DTAC and DTAB are used as cationic agents, respectively. In the case of surfactant-ssDNA particles, and for a concentration of ssDNA equal to 5 μg mL^{-1}, the degree of haemolysis is 20%, 50% and 70%, when DTATf, DTAC and DTAB are used as surfactants, respectively. The haemolysis response found in these DNA gel particles can be correlated with differences in the apparent degree of counter ion dissociation in these surfactants from the corresponding micelles [98-100].

4.2. Cytotoxicity

Cytotoxicity plays a critical role in the efficiency of the delivery vectors. In order to deliver the DNA into the cells, the cationic particles bind to the cell surface by

electrostatic interaction, promote endocytosis and release the genetic material inside the cell. Unfortunately, while high concentrations of the delivery agents imply an increased chance of the DNA penetrating the cell nucleus, they can also interfere with physiological processes within the cell, inducing cell death. Thus, present research is aimed at designing gene delivery agents that are able to deliver DNA into the cells with minimal toxicity [101].

Despite the significant scientific interest and promising potential of the particulate materials, the safety of these systems remains a growing concern, considering that biological applications of particles could lead to unpredictable effects. Currently, there are no specific testing requirements for nanotechnology products, and therefore, researchers took liberal approaches to studying toxicity [102-103]. Moreover, it is worth noting that, because of the expense of animal testing in toxicology and pressure from both the general public and government to develop alternatives to *in vivo* testing, *in vitro* cell-based models may be more attractive for preliminary testing of nanomaterials [104]. The prediction of toxicity is difficult, but cytotoxicity screening, which is routinely used in drug screening, gives a good indication of potential adverse effects in cells. Rapid, sensitive and reliable bioassays are required to examine the toxicity. Established cell lines are useful alternative test systems for this kind of toxicological studies [105]. However, they must be chosen with care with regard to their origin [106]. Cytotoxicity assays are among the most common *in vitro* endpoints used to predict the potential toxicity of a substance in a cell culture [107]. Cell damage is manifested in several ways, including mitochondrion and plasma membrane dysfunction and fluctuating intracellular reduction capacity [108.Current standard approaches to gauge the degree of cell damage include assays that measure various aspects of cell viability, such as metabolic activity and plasma membrane integrity. The MTT reduction assay which is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide by cellular dehydrogenases, is among the most commonly used endpoints. This method measures the reduction of MTT salt to a coloured insoluble formazan in active mitochondria in viable cells and also, in certain cases, outside the mitochondria [109-110].

The first approach on the determination of the cytotoxic response of DNA gel particles have been carried out on protein-DNA gel particles formed using two different approaches by mixing double-stranded DNA (dsDNA) with lysozyme (LS) and

protamine sulfate (PS) mixtures. The interaction of these protein-DNA gel particles and their components with non-tumour (3T3 fibroblast) and tumour (HeLa) cell lines has been determined, using the imposed variations in protein composition and the size of the final particles, as a consequence of the different preparation method, as controlling parameters [64].

Assessing the capacity of live cells to metabolise a tetrazolium colourless salt to a blue formazan (MTT assay) were used to perform indirect measurements of cell viability. Dose–response curves for each protein, determined by MTT assays using tumour cell line HeLa and non-tumour cell line 3T3 fibroblasts, are given in Fig. 10. The cytotoxicity assays were performed in the concentration range 50 and 2000 μg mL^{-1}. Although it is thought that proteins are biocompatible and nontoxic compounds, our results have revealed that, as with other cationic derivatives, LS and PS displayed concentration-dependent toxicity towards cells *in vitro*. LS showed low cytotoxicity towards 3T3 cells, which displayed viability in the range 81% to 100% as determined by the MTT assay (Fig. 11a) at the tested protein concentration range. In the case of PS, viability changed from 7% to 100% depending on the concentration. These experiments enabled us to define the protein concentration required to inhibit cell growth by 50% compared with an untreated control (IC_{50}). In the case of LS, its IC_{50} was found to be higher than 2000 μg mL^{-1} in both cell lines. For PS, it was found to be 140 and 250 μg mL^{-1} for 3T3 and HeLa cell lines, respectively (Fig. 11b). These differences could be attributed to differences in the binding characteristics of these two proteins, with different total charge and linear charge density: LS is a globular protein that has a net charge of + 9 at neutral pH, whereas PS is a highly positively charged linear protein with an overall charge of +21.

[Fig. 11 here]

Cell culture studies have greatly increased the understanding of cellular functions and complex signalling pathways and have been routinely used for toxicity screening of new compounds. Based on the assumption that a decrease in cellular vitality reduces physiological function, cellular products and cell number, cytotoxicity screening assays that measure enzymatic activities or cell products have been carried out. These assays perform reliably with chemical compounds but can produce false results by interference with particulate systems. Most examples have been published for the MTT assay. Nanomaterials interfere with the assay by light absorbance, reduction of the tetrazolium salt, and binding of the formazan salt [111].

Interactions of nanomaterials assays include interference by adsorption of dyes [112], absorbance [113], fluorescence [114], binding of proteins [115], dye degradation [116] and dye formation [112], among others.

In each of these cases the underlying phenomena involve some interaction between nanomaterials and organic small-molecule solutes in multicomponent biological phases. Less attention has been paid to the fundamental interactions of nanomaterials with *small* biomolecules in complex physiological fluid phases. Inspired on the observation that small quantities of nanotubes removed the colour associated with the pH indicator dye, phenol red, in cell culture medium [117], it has been described that nanotube adsorption causes profound changes in the composition of the medium, and thus has the potential to influence *in vitro* cell behaviour through an indirect mechanism that does not involve physical interaction between nanotubes and target cells [118].

Previous studies in our laboratory have verified the absorption of the culture media by the particles when 3T3 and HeLa cell lines were incubated in the presence of some surfactant-DNA gel particles prepared by the dropwise addition method. In this case, the obtained cell viabilities were close to 5% (results not published). Although the IC_{50} values of the corresponding surfactants in solution were not very high (with values around 10 $\mu g\ mL^{-1}$), this low cell viability may be correlated with the physicochemical properties of these DNA gel particles. Even though no systematic studies on the composition of the medium have been carried out, the observed swelling behaviour of some of these DNA gel particles point out the effect of adsorption of essential micronutrients from cell culture medium. Therefore, toxicity data must be interpreted in the context of the physicochemical characteristics of the particulate systems.

Concerning the protein-DNA gel particles prepared by the dropwise addition method, visual inspection of the corresponding plates exhibited no evident changes on the volume and characteristics of the culture media when 3T3 and HeLa were incubated in the presence of individual protein-DNA gel particles during 24h. Although these particles are several magnitudes larger than cells and cannot be internalized as a whole, this study demonstrates that the physicochemical properties of these protein-DNA gel particles may not affect their cytotoxic characterization under standard protocols. As has been already pointed, an important implication of the changes in the composition of the medium, as assessed by commonly used endpoints, such as cell viability, DNA damage,

and apoptosis, may be mistakenly attributed to direct toxicity when in fact it is a secondary effect of adsorption of essential micronutrients from cell culture medium.

Cell viabilities of up to 80% were observed in almost all compositions when the cytotoxicity of the corresponding protein-DNA gel particles was determined in both cell lines (Fig. 12a). However, cell viabilities were always lower than 10-20% when cells were incubated in the presence of pure and mixed protein solutions (Fig. 12b).

[Fig. 12 here]

In previous studies in our lab, we have prepared nano-/micro-sized DNA gel particles by nebulisation of DNA solutions (either single- (ssDNA) or double-stranded (dsDNA)) into an oppositely charged surfactant or protein solution [77]. FM studies suggest that the formation of the particles was carried out with conservation of the secondary structure of the nucleic acid molecules. SEM on freeze-dried and Au-shadowed samples showed a distribution of virtually spherical particles. It was found that, in addition to the size of the initial DNA droplets, the cationic agent is a controlling parameter of the particle size. In the case of protein-DNA gel particles, LS-DNA gel particles showed diameters around 10 μm whereas the size of PS-DNA was around 400 nm (see Table 2). In a recent study, small-sized mixed protein-DNA gel particles were prepared for the first time, and their cytotoxicity was evaluated. Although the particle dispersions were studied without further purification, and both free protein and protein-DNA gel particles were present in the obtained dispersions, the cytotoxic responses shown in Fig. 13a were observed to be significantly different to that observed with proteins in solution (Fig. 13b). As expected, the cytotoxicity of the obtained DNA particles resulted lower than that observed with the corresponding protein in solution. Only the LS system showed an irregular behaviour.

[Fig. 13 here]

The observed differences in cytotoxicity may be correlated with differences in protein-DNA complexation in these systems. Consequently, we determined the initial amount of protein in the media, as well as the amount of protein remaining in the dispersion containing the protein-DNA gel particles formed by the nebulisation method. From these values, the degree of complexation in the different systems was determined.

Fig. 13c shows the evolution of the degree of complexation according to the LS/PS ratio. In the case of the protein-DNA gel particles, it was expected that the higher the degree of complexation, the smaller the amount of protein that would be released in solution, an amount which would be able to interact with the cells and reduce their viability. Independently of the cell line response, the differences in the degree of complexation were in agreement with the observed trend in cell viabilities (Fig. 13d).

Differences in cytotoxicity between protein-DNA particles prepared by the dropwise addition method and the nebulisation method could be related to differences in the kinetics of dissolution/release profiles. Studies of DNA release from protein-DNA particles formed by the dropwise addition method have demonstrated that these particles can present DNA release profiles of up to 1,000 h, confirming the stability of these protein-DNA gel particles [63]. In the present study, the stability of these particles in the culture medium was also confirmed. Supported by visual observation, the particles remained present in the well plate after 24h of incubation. This behaviour and the fact that the observed cytotoxicity was almost independent of the protein composition (Fig. 12a) corroborate this argument. Although the profiles of dissolution/release of the protein-DNA gel particles prepared by the nebulisation method have not yet been determined, it is expected that smaller particles will show faster dissolution profiles. As a consequence of the protein release, a more composition-dependent cytotoxic response compared with that observed at large-sized particles could be awaited. In the case of these small-sized protein-DNA particles the cytotoxic responses were strongly dependent on the protein composition (Fig.13a).

The preparation of the protein-DNA gel particles by the nebulisation method enabled us to obtain particle dispersions in order to evaluate the effect of the concentration (in this study, expressed as protein concentration). Observation of IC_{50}-values showed that the two cells lines were markedly different in sensitivity to the cytotoxic effects of these protein-DNA particles (Fig. 14 and Table 3). Except in the case of the pure systems, which displayed identical cytotoxicity in both cell lines, the tumour cell line HeLa was more sensitive to the deleterious effects of the mixed protein-based particles than 3T3 fibroblasts (significant differences between 3T3 and HeLa for the same conditions are indicated in Table 3 with an asterisk). Consequently, the mixing of proteins had a clear modulating effect on the relative cytotoxicity of these systems towards tumour and non-tumour cell lines.

[Fig. 14 here]

5. Summary

A general understanding of interactions between DNA and oppositely charged agents provides a basis for developing novel DNA gel particles. The adsorption strength, which is tuned by varying the structure of the cationic agent, allows the control of the spatial homogeneity of the gelation process, producing either a homogeneous DNA matrix or different DNA reservoir devices. It was shown that DNA was effectively entrapped in the assayed cationic surfactant, protein and polysaccharide solutions, protecting its secondary structure. When the DNA gel particles are inserted in a medium, different responses occur: swelling or deswelling, dissolution, and DNA release. Controlling the magnitude of the DNA release and achieving controlled release systems was accomplished by changing the composition in the cationic solutions where particles were formed. Modulation of the superficial hydrophobicity of the DNA gel particles, as a consequence of the imposed compositions, may govern the posterior adsorption of plasma proteins and influence the overall bioavailability of the systems.

One drawback of the DNA gel particles, in toxicological terms, is the need for a cationic compound, which may cause some cell damage. However, our results indicate that the effect of the cationic agent can be modulated when administered in a DNA gel system, rather than in an aqueous solution. Unlike delivery in an aqueous solution, the cytotoxicity of the cationic system can be reduced when the opposite charges between the cationic compounds and DNA spontaneously result in complexation due to electrostatic interactions. The magnitude of DNA entrapment can be controlled and controlled release systems achieved through the formation of a DNA-oppositely charged complex network giving rise to these DNA gel particles. The decrease in toxicity as well as the formation of a releasable high DNA content reservoir renders these DNA gel particles promising DNA vehicles for use as a nonviral gene delivery system.

6. Prospects

Over the last two decades, gene therapy has brought human medical prospect into a new phase, whereby genetic defects on cells can be regulated and also a range of diseases can be prevented. DNA-based molecules are being employed to prevent, treat, and cure diseases by changing the expression of genes that are responsible for the pathology. Since its inception, plasmid-DNA-mediated gene therapy has seen significant growth and brings fruitful clinical trials. However, the major underlying challenge is the development of a carrier system that must have the capacity to enter the cells of interest, to protect nucleic acids from nuclease degradation, to escape the endocytic pathway and reach the cytosol, to dissociate and release the DNA, and to facilitate the integration and activity of the transferred DNA inside the nucleus. In the absence of cell division, and additional limiting step is the translocation of DNA through the nuclear envelope. Some recent works have turned to investigate the nucleus entry problem [119]. Evidently nuclear pore complexes (NPC) act as gateway for macromolecular traffic between the cytoplasm and the nucleus. Short nucleic acids such as oligonucleotides can diffuse freely through the pore. Larger molecules need to be actively transported by a cargo protein that carries a nuclear nuclear localization signal (NLS). Endogenous nuclear proteins, whose natural functions are to condense DNA and which possess one or more NLS sequences, are interesting candidates to mediate nuclear translocation. Among these are proteins from the high mobility group (HMG), histones (especially H1), and protamines that were shown to enhance in vitro transfection efficiency properties [120-122]. We are engaged in current work focusing on the use of nuclear protein on the formation of DNA gel particles based on associative phase separation and interfacial diffusion [123].

An alternative promising approach comes from the observation that glycoproteins lacking NLS are able to enter the nucleus. Oligosaccharides are presumably recognized by lectins (a component of the nuclear pore complex). If bound to DNA, these oligosaccharides can facilitate its transport through the nuclear envelope. Recent research has been focused on the application of the pure chitosan oligosaccharide lactate or in combination with protamine sulfate as promising DNA carriers [75].

Colloidal carriers have offered the opportunity to design surface properties to enable them traverse biological barriers such as skin, mucous barriers, and leaky vasculature.

The smart design of the colloidal carrier can protect DNA-based molecules from deleterious degradation, and may provide sustained release of payload in a therapeutically advantageous fashion. Although these carriers may offer various advantages over conventional drug-delivery systems, their safety should not be ignored. The toxicity of these nanomaterials may be due to their large surface area. A continuous effort focused on improving safety, feasibility, and efficacy of colloidal carriers for DNA gene therapy is required.

Abbreviations

ALA: arginine-N-lauroyl amide dihydrochloride

CHIT: chitosan

CL: chitosan lactate

CTAB: cetyltrimethylammonium bromide

DDAB: didodecyldimethylammonium bromide

DTAB: dodecyltrimethylammonium bromide

DTAC: dodecyltrimethylammonium chloride

DTATf: dodecyltrimethylammonium trifluoromethane sulfonate

LAM: N^{α}-lauroyl-arginine-methyl ester hydrochloride

LS: lysozyme

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PS: protamine sulfate

Acknowledgements

This work was financially supported by the Project SFRH/BPD/47229/2008 from Fundação para a Ciência e a Tecnologia (FCT), and the Projects CTQ2009-14151-C02-02 and MAT2012-38047-C02-01 from the Spanish Ministry of Science and Innovation. M.C. Morán acknowledges the support of the MICINN (Ramon y Cajal contract RyC 2009-04683).

References

[1] Houk B, Martin R, Hochchaus G, Hughes, J. Pharmacokinetics of plasmid DNA in rats. Pharm Res 2001;18(1): 67-74.

[2] Dias R S, Lindman B (Eds). DNA Interactions with Polymers and Surfactants. 2008; Wiley Interscience, New Jersey.

[3] Ledley F D, Pharmaceutical Approach to Somatic Gene Therapy. Pharm Res 1996; 13 (11): 1595-1614.

[4] O´Hagan D T, Singh M, Ulmer J B , Microparticles for the delivery of DNA vaccines. Immunol Rev 2004; 199(1): 191-200.

[5] Patil S D, Rhodes D G, Burgess D J, DNA-based therapeutics and DNA delivery systems: A comprehensive review. AAPS Journal 2005; 7 (1): E61-E77.

[6] Caplen N J, Alton E W F W, Mddleton P G, Dorin J R, Stevenson B J, Gao X, Durham S R, Jeffery P K, Hodson M E, Coutelle C, Huang L, Porteous D J, Williamson R, Geddes D M, Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis, Nature Med 1995; 1 (1): 39-46.

[7] Cotton M, Wagner E, Non-viral approaches to gene therapy. Curr Opin Biotechnol 1993; 4 (6): 705–710.

[8] Singhal A, Huang L, Gene transfer in mammalian cells using liposomes as carriers, in: J.A. Wolff (Ed.), Gene as a drug. Therapeutics: Methods and Applications of Direct Gene, Birkhauser, Boston, 1994.

[9] Schonfield J P, Caskey C T, Non-viral approaches to gene therapy. Br Med J 1995; 51: 56-71.

[10] Truong-Le V L, August J T, Leong K W, Controlled gene delivery by DNA-gelatin nanospheres. Hum Gene Ther 1998; 9 (12): 1709-1717.

[11] Leong K W, Mao H Q, Truong-Le V L, Roy K, Walsh S M, August J T, DNA-polycation nanospheres as non-viral gene delivery vehicles. J Controlled Release 1998; 53 (1-3): 183-193.;

[12] Drury J L, Mooney D J, Hydrogels for tissue engineering: scaffold design variables and applications. Biomaterias 2003; 24(24): 4337-4351.

[13] Langer R, Peppas N A, Advances in Biomaterials, Drug Delivery, and Bionanotechnology. AIChE Journal 2003; 49: 2990-3006.

[14] Muzzarelli R A A, Muzzareli C, Chitosan chemistry: Relevance to the biomedical sciences. Adv Polym Sci 2005; 186: 151-209.

[15] Rinaudo M, Characterization and Properties of Some Polysaccharides Used as Biomaterials. Macromol Symp 2006; 245-246 (1): 549-557.

[16] Bryant S J, Anseth K S, Controlling the spatial distribution of ECM components in degradable PEG hydrogels for tissue engineering cartilage. J Biomed Mater Res 2003; 64A (1): 70-79.

[17] Burdick J A, Chung C, Jia X, Randolph M A, Langer R, Controlled degradation and mechanical behavior of photopolymerized hyaluronic acid networks. Biomacromolecules 2005; 6 (1): 386-391.

[18] Levesque S G, Shoichet M S, Synthesis of enzyme-degradable, peptide-crosslinked dextran hydrogels . Bioconjugate Chem 2007 18: 874-885.

[19] Metters A, Hubbell J, . Network formation and degradation behavior of hydrogels formed by Michael-type addition reactions. Biomacromolecules 2005; 6; 290- 301.

[20] Rydholm A E, Reddy S K, Anseth K S, Bowman C N, Controlling Network Structure in Degradable Thiol-Acrylate Biomaterials to Tune Mass Loss Behavior. Biomacromolecules 2006; 7; 2827-2836.

[21] Skarja G A, Woodhouse K A, In vitro degradation and erosion of degradable, segmented polyurethanes containing an amino acid-based chain extender. J Biomater Sci Polym Ed 2001; 12 (8) 851-873.

[22] Timmer M D, Ambrose C G, Mikos A G, In vitro degradation of polymeric networks of poly(propylene fumarate) and the crosslinking macromer poly(propylene fumarate)-diacrylate . Biomaterials 2003; 24 (4): 571-577.

[23] Yu L M Y, Kazazian K, Shoichet M S, Peptide surface modification of methacrylamide chitosan for neural tissue engineering applicationsJ Biomed Mater Res 2007; 82A (1) 243-255.

[24] Gupta D; Tator C H, Shoichet M S, Fast-gelling injectable blend of lyaluronan and methylcellulose for intrathecal, localized drug delivery to the injured spinal cord, Biomaterials 2006; 27 : 2370-2379.

[25] Kong H J, Alsberg E, Kaigler D, Lee K Y, Mooney D J, Controlling degradation of hydrogels via the size of crosslinked junctions. AdV Mater 2004, 16(21): 1917-1921.

[26] Lynn D M, Layers of opportunity: nanostructured polymer assemblies for the delivery of macromolecular therapeutics. Soft Matter 2006; 2: 269-273.

[27] Tate M C, Shear DA, Hoffman S W Stein D G, LaPlaca M C, Biocompatibility of methylcellulose-based constructs designed for intracerebral gelation following experimental traumatic brain injury. Biomaterials 2001, 22 (10): 1113-1123.

[28] Lapitsky Y, Zahir T, Shoichet M S, Modular Biodegradable Biomaterials from Surfactant and Polyelectrolyte Mixtures. Biomacromolecules 2008; 9: 166-174.

[29] Piculell L, Lindman B, Association and segregation in aqueous polymer/polymer, polymer/surfactant, and surfactant/surfactant mixtures: similarities and differences. Adv Colloid Interface Sci 1992; 41: 149–178.

[30] Thalberg K, Lindman B, Polymer-surfactant interactions-recent developments, in Interactionsof Surfactants with Polymers and Proteins, ed. D. E. Goddard and K. P. Ananthapadmanabhan, CRC Press, Boca Raton, 1993, pp 203–276.

[31] Ober C K, Polyelectrolyte–Surfactant Complexes in the Solid State: Facile building blocks for self-organizing materials†Wegner G, Adv Mater 1997; 9 (1) :17-31.

[32] Carnali J O, (Polymer/polymer)-like phase behavior in the system tetradecyltrimethylammonium bromide/sodium polyacrylate/water.Langmuir 1993; 9 (11); 2933–2941.

[33] Wang Y L, Kimura K, Huang Q, Dubin P L, Jaeger W, Effects of Salts on Polyelectrolyte-Micelle Coacervation, Macromolecules 1999; 32 (21): 7128–7134.

[34] Wang Y L, Kimura K, Dubin P L, Jaeger W, Polyelectrolyte-Micelle Coacervation: Effects of Micelle Surface Charge Density, Polymer Molecular Weight, and Polymer/Surfactant Ratio Macromolecules 2000; 33 (9): 3324–3331.

[35] Ilekti P, Martin T, Cabane B. Piculell L, Effects of Polyelectrolytes on the Structures and Interactions of Surfactant Aggregates. J Phys Chem B 1999; 103 (45) : 9831–9840.

[36] Marques E F, Regev O, Khan A, Miguel M G. Lindman B, Interactions between Catanionic Vesicles and Oppositely Charged Polyelectrolytes-Phase Behavior and Phase Structure. Macromolecules 1999; 32 : 6626–6637.

[37] Swanson-Vethamuthu M, Dubin P L, Almgren M, Li Y, Cryo-TEM of Polyelectrolyte–Micelle Complexes. J Colloid Interface Sci 1997; 186 (2): 414–419.

[38] Julià-Ferrés M R, Erra Serrabasa P, Muñoz Lirón I, Ayats Llorents A, Procedure for preparing capsules and for encapsulation of substances, Patent No. ES2112150, 1998, Spain.

[39] Babak V G, Merkovich E A, Galbraikh L S, Shtykova E V, Rinaudo M, Kinetics of diffusionally induced gelation and orderd nanostructure formation in surfactant-polyelectrolyte complexes formed at water/water emulsion type interfaces. Mendeleev Commun 2000; 3: 94–95.

[40] Stamberg J, Dautzenberg H, Loth F, Benes M, Kuhn A,Patent No. 218372 B 15/06, 1983, Germany.

[41] Nishimura K, Nishimura S, Seo H, Nishi N, Tokura S, Azuma I, with multi-porous beads prepared from partially deacetylated chitin. .J Biomed Mater Res 1986; 20: 1359–1372.

[42] Philipp B, Dautzenberg H, Linow K J, Koetz J, Dawydoff W, Polyelectrolyte complexes-recent developments and open problems. Prog Polym Sci 1989; 14 (1) : 91–172.

[43] Lapitsky Y, Kaler E W, Formation of Surfactant and Polyelectrolyte Gels in Aqueous Solutions. Colloids Surf A 2004; 250 (1-3): 179–187.

[44] Lapitsky Y, Eskuchen W J, Kaler E W, Surfactant and Polyelectrolyte Gel Particles that Swell Reversibly. Langmuir 2006; 22: 6375-6379.

[45] Lapitsky Y, Kaler E W, Formation and Structural Control of Surfactant and Polyelectrolyte Gels. Colloids Surf A 2006; 282-283: 118-128.

[46] Lapitsky Y, Kaler E W, Surfactant and Polyelectrolyte Gel Particles for Encapsulation and Release of Aromatic Oils. Soft Matter 2006; 2: 779-784.

[47] Costa D, Morán M C, Miguel M G, Lindman B, Cross-linked DNA Gels and Gel Particles, in DNA Interactions with Polymers and Surfactants, Dias R S, Lindman B (Eds) 2008; Wiley Interscience, New Jersey.

[48] Lindman B, Dias R S, Miguel M G, Morán M C, Costa D. Manipulation of DNA by Surfactants, in Highlights in Colloid Science, Platikanov D, Exerowa D (Eds) 2009; Wiley-VCH, Weinheim.

[49] Morán M C, Miguel M G, Lindman B. DNA gel particles. Soft Matter 2010;6 (14) 3143-3156.

[50] Dias R, Morán M C, Costa D, Miguel M, Lindman B, DNA-Surfactant Systems: Particles, Gels and Nanostructures, in Nano-Science: Colloidal and Interfacial Aspects, Starov V. M (Ed) 2010; Taylor & Francis.

[51] Morán M C, Costa D, Miguel M G, Lindman B, DNA release from cross-linked DNA gels and DNA gel particles, in Surfactant Science and Technology: Retrospects and Prospects, Romsted, L. (Ed) 20013; Taylor and Francis.

[52] Morán M C, Miguel M G, Lindman B. DNA gel particles: Particle preparation and release characteristics. Langmuir 2007; 23 (12): 6478-81.

[53] Morán M C, Miguel M G, Lindman B. Surfactant-DNA gel particles: Formation and release characteristics. Biomacromolecules 2007; 8(12): 3886-92.

[54] Morán, M. C, Alonso T, Lima F S, Vinardell M P, Miguel M G, Lindman B. Counter-ion effect on surfactant-DNA gel particles as controlled DNA delivery systems. Soft Matter 2012; 8 (11): 3200-11.

[55] Morán M C, Infante M R, Miguel M G, Lindman B, Pons R. Novel biocompatible DNA gel particles. Langmuir 2010; 26 (13): 10606-13.

[56] Morán M C, Miguel M G, Lindman B. DNA gel particles from single and double-tail surfactants: supramolecular assemblies and release characteristics. Soft Matter 2011; 7 (5): 2001-10.

[57] Morán M C, Pinazo A, Pérez L, Clapés P, Angelet M, García M T, Vinardel M P, Infante M R. "Green" amino acid-based surfactants. Green Chem 2004; 6 (5): 233-40.

[58] Infante M R, Pérez L, Morán M C, Pons R, Mitjans M, Vinardell M P, García M T, Pinazo A. Biocompatible surfactants from renewable hydrophiles. Eur J Lipid Sci Technol 2010; 112 (1):110-21.

[59] Record M T J, Anderson C F, Lohman T M. Thermodynamic Analysis of Ion Effects on the Binding and Conformational Equilibria of Proteins and Nucleic Acids: The Roles of

Ion Association or Release,Screening and Ion Effects on Water Activity. Q Rev Biophys II 1978; 11: 103-78.

[60] Sharp K A, Friedman R A, Misra V, Hecht J, Honig B. Salt effects on polyelectrolyte-ligand binding: Comparison of Poisson-Boltzmann and limiting law counterion binding models. Biopolymers 1995; 36 (2): 245-62.

[61] Palkar S A, Lenhoff A M. Energetic and Entropic Contributions to the Interaction of Unequal Spherical Double Layers. J Colloid Interface Sci 1994; 165 (1) 177-94.

[62] Koynova R, Tenchov B. Cationic phospholipids: structure–transfection activity relationships. Soft Matter 2009; 5:3187-200.

[63] Morán M C, Ramalho A, Pais A A C C, Miguel M G, Lindman B. Mixed protein carriers for modulating DNA release. Langmuir 2009; 25 (17): 10263-70.

[64] Morán M C, Nogueira D R, Vinardell M P, Miguel M G, Lindman B. Mixed protein-DNA gel particles for DNA delivery: Role of protein composition and preparation method on biocompatibility. Int J Pharm 2013; 454: 192-203.

[65] Ibrahim H R, Higashiguchi S, Juneja L R, Kim M, Yamamoto T. A structural phase of heat-denatured lysozyme with novel antimicrobial action. J Agric Food Chem 1996; 44: 1416–23.

[66] Gaweda S, Morán M C, Pais A A C C, Dias R S, Schillén K, Lindamn B, Miguel M G. Cationic agents for DNA compaction. J Colloid Interface Sci 2008; 323 (1): 75-83.

[67] Pinto M F V, Morán M C, Miguel M G, Lindman B, Jurado A S, Pais A A C C. Controlling the morphology in DNA condensation and precipitation. Biomacromolecules 2009; 10(6): 1319-23.

[68] Brewer L, Corzett M, Balhorn R. Condensation of DNA by spermatid basic nuclear proteins. J Biol Chem 2002; 277 (41): 38895-900.

[69] Saito G, Amidon G L, Lee K D. Enhanced cytosolic delivery of plasmid DNA by a sulfhydryl-activatable listeriolysin O/protamine conjugate utilizing cellular reducing potential Gene Ther 2003; 10: 72-83.

[70] Kas H S. Chitosan: Properties, preperation and application to microparticulate systems. J Microencapsulation 1997; 14: 689-711.

[71] Lee M, Nah J W, Kwon Y, Koh J J, Ko K S, Kim S W. Water-soluble and low molecular weight chitosan-based plasmid DNA delivery. Pharm Res 2001; 18 (4): 427-31.

[72] Maclauglin F C Mumper R J, Wang J, Tagliaferri J M Gill I, Hinchcliffe M, Rolland A P. Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. J Controlled Release 1998; 56 (1-3) 259–72.

[73] Mao H Q, Roy K, Troung-Le V L, Janes K A, Lin K, Wang Y, August J T, Leong K W. hitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. J Controled Release 2001; 70 (3): 399-421.

[74] Morán M C, Laranjeira T, Ribeiro A, Miguel M G, Lindman B. Chitosan-DNA particles for DNA delivery: Effect of chitosan molecular weigth on formation and release characteristics. J Dispersion Sci Technol 2009; 30 (10) 1494-99.

[75] Morán M C, Pérez E, Vinardell M P. Chitosan-based DNA gel particles for modulating DNA release. Soft Matter 2013 (submitted).

[76] Rejman J, Oberle V, Zuhorn I S, Hoekstra D, Size-dependent internalization of particles via the pathways of clathrinand caveolae-mediated endocytosis Biochem J 2004; 377: 159-169.

[77] Morán M C, Baptista F R, Ramalho A, Miguel M G, Lindman B. DNA gel nanoparticles: preparation and controlling the size. Soft Matter 2009; 5: 2538-42.

[78] Rigler R, Killander D, Bolund L, Ringertz N. R. Cytochemical characterization of deoxyribonucleoprotein in individual cell nuclei. Exp Cell Res 1969; 55 (2): 215-24.

[79] Ichimura S, Zama M, Fujita H. Quantitative determination of single-stranded sections in DNA using the fluorescent probe acridine orange. Biochim Biophys Acta 1971; 240: 485-95.

[80] Peacocke A R. The interaction of acridines with nucleic acids, in Acridines, Acheson, R. M. (Eds) 1973; Interscience Publishers, New York.

[81] Darzynkiewicz Z, Traganos F, Sharpless I, Melamed M R. Thermal denaturation of DNA in situ as studied by acridine orange staining and automated cytofluorometry. Exp Cell Res 1975; 90 (2): 411-28.

[82] Cser A, Nagy K, Biczók L. Fluorescence lifetime of Nile Red as a probe for the hydrogen bonding strength with microenvironment. Chem Phys Lett 2002; 360 (5-6) 473-78.

[83] Joshi M D, Müller R H. Lipid nanoparticles for parenteral delivery of actives. Eur J Pharm Biopharm 2009; 71 (2): 161–72.

[84] Gessner A, Waicz R, Lieske A, Paulke B R, Mäder K, Müller R H. Nanoparticles with decreasing surface hydrophobicities: influence on plasma protein adsorption. Int J Pharm 2000; 196 (2): 245–49.

[85] Aggarwal P, Hall J B, McLeland C B, Dobrovolskai M A, McNeil S E. Nanoparticle interaction with plasma proteins as it related to particle biodistribution, biocompatibility and therapeutic efficacy. Advanced Drug Delivery Reviews 2009; 61(6): 428-37.

[86] Owens D E, Peppas N A. Opsonization, biodistribution, and pharmacokinetics of polymer nanoparticles. Int J Pharm 2006; 307 (1) 93–102.

[87] Carstensen H, Muller R H, Muller B W. Particle-size, surface hydrophobocity and interaction with serum of parenteral fat emulsions and model-drug carriers as parameters related to res uptake. Clin Nutr 1992; 11 ( ): 289–97.

[88] Muller R H, Wallis K H, Troster S D, Kreuter J. In vitro characterization of poly (methyl-methacrylate) nanoparticles and correlation to their in vivo fate. J Control Release 1992; 20 (3) 237–46.

[89] Norman M E, Williams P, Illum L. Human serum-albumin as a probe for surface conditioning (opsonization) of block-copolymer-coated microspheres. Biomaterials 1992; 13 (12) 841–49.

[90] Cedervall T, Lynch I, Foy M, Berggard T, Donnelly S C, Cagney G, Linse S, Dawson K A. Detailed identification of plasma proteins adsorbed on copolymer nanoparticles. Angew Chem Int Ed Engl 2007; 46 (30):5754–56.

[91] Mielke M, Zimelh R. Measures to determine the hydrophobicity of colloidal polymers. Progr Colloid Polym Sci S 2001; 117: 56-62.

[92] Müller R H, Davis S S, Illum L, Mak E. Particle charge and surface hydrophobicity of colloidal drug carriers. In : G. Gregiruadus, J. Senior, G.Poste (Eds.)1986; Targeting of Drugs with Synthetic Systems. Plenum, p. 239.

[93] Müller R H. Surface hydrophobocity-determination by rose bengal (rb) adorption methods. In: R. H. Müller, W. Mehnert (Eds), 1997; Particle and Surface Characterisation Methods. Wissenschaftliche Verlagsgesellschaft, Stuttgartm pp. 215-228.

[94] Bhowmik B B, Ganguly P. Photophysics of xanthene dyes in surfactant solution. Spectrochim Acta A 2005; 61 (9): 1997-2003.

[95] Sheetz M P, Singer S J. Biological Membranes as Bilayer Couples. A Molecular Mechanism of Drug-Erythrocyte Interactions. Proc Natl Acad Sci USA1 1974; 71 (11) 4457-61.

[96] Isomaa B, Hägerstrand H, Paatero G. Shape transformations induced by amphiphiles in erythrocytes. Biochim Biophys Acta 1987; 899 (1): 93-103.

[97] Vinardell M P, Infante M R. The relationship between the chain length of non-ionic surfactants and their hemolytic action on human erythrocytes. Comp Biochem Physiol Part C 1999; 124 (2): 117-120.

[98] Bales B L, Benrraou M, Zana R. Characterization of Micelles of Quaternary Ammonium Surfactants as Reaction Media I: Dodeclytrimethylammonium Bromide and Chloride. J Phys Chem B 2002; 106: 1926-39.

[99] Perger T M, Bester-Rogac M. hermodynamics of micelle formation of alkyltrimethylammonium chlorides from high performance electric conductivity measurements. J Colloid Interface Sci 2007; 313 (1): 288–95.

[100] Lima F S, Maximiano F A, Cuccovia I M, Chaimovich H. Surface activity of the triflate ion at the air/water interface and properties of N,N,N-trimethyl-N-dodecylammonium triflate aqueous solutions. Langmuir 2011; 27 (8): 4319-23.

[101] Rao G, Yadava P, Hughes J. Rationally designed synthetic vectors for gene delivery. The Open Drug Delivery Journal 2007; 1: 7-19.

[102] Boverhof D R, David R M. Nanomaterial characterization: considerations and needs for hazard assessment and safety evaluation. Anal Bioanal Chem 2010; 396 (3): 953–61.

[103] Robbens J, Vanparys C, Nobels I, Blust R, Hoecke K V, Janssen C, Schamphelaere K D, Roland K, Blanchard G, Silvestre F, Gillardin V, Kestemont P, Anthonissen R, Toussaint O, Vankoningsloo S, Saout C, Alfaro-Moreno E, Hoet P, Gonzalez L, Dubruel P, Troisfontaines P. Eco-, geno-, and human toxicology of bio-active nanoparticles for biomedical applications. Toxicology 2010; 269 (2-3) 170-81.

[104] Hillegass J M, Shukla A, Lathrop S A, MacPherson M B, Fukagawa N K, Mossman BT. Assessing nanotoxicity in cells in vitro. WIREs Nanomed Nanobiotechnol 2009; 2: 219–31.

[105] Crespi C L. Xenobiotic-metabolizing human cells as tools for pharmacological and toxicological research. Adv Drug Res 1995; 26: 179-235.

[106] Jondeau A, Dahbi L, Bani-Estivals M H, Chagnon M C. Evaluation of the sensitivity of three sublethal cytotoxicity assays in human HepG2 cell line using water contaminants. Toxicology 2006; 226 (2-3) 218-28.

[107] Martinez V, Corsini E, Mitjans M, Pinazo A, Vinardell M P. Evaluation of eye and skin irritation of arginine-derivative surfactants using different in vitro endpoints as alternatives to the in vivo assays. Toxicol Lett 2006; 164 (3): 259-67.

[108] Kim H, Yoon S C, Lee T Y, Jeong D. Discriminative cytotoxicity assessment based on various cellular damages. Toxicol Lett 2009; 184 (1) 13–7.

[109] Berridge MV, Herst PM, Tan A S. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol. Annu. Rev. 2005; 11: 127–52.

[110] Liu Y, Peterson DA, Kimura H, Schubert D. Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. J Neurochem 1997; 69 (2): 581–93.

[111] Worle-Knirsch J M, Pulskamp K, Krug H F, Carbon nanotubes hoax scientists in viability assays. Nano Lett 2006; 6 (6): 1261-1268.

[112] Monteiro-Riviere N A, Inman A O, Zhang L W, Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. Toxicol. Appl. Pharmacol., 2009, 234 (2), 222-235.

[113] Zhang LW, Zeng L, Barron AR, Monteiro-Riviere NA, Biological interaction of functionalized single-wall carbon nanotubes in human epidermal keratinocytes. Int J Toxicol. 2007; 26:103−113.

[114] Stone V, Johnston H, Schins R P F, Development of in vitro systems for nanotoxicology: methodological considerations. Crit Rev Toxicol 2009; 39 (7): 613-626.

[115] Hurt R H, Monthioux M, Kane A, Toxicology of carbon nanomaterials: status, trends, and perspectives on the special issue. Carbon 2006; 44, 1028–1033.

[116] Hasnat MA, Uddin MM, Samed AJF, Alam SS, Hossain S. Adsorption and photocatalytic decolorization of a synthetic dye erythrosine on anatase TiO_2 and ZnO surfaces.J Hazard Mater.2007;147:471–477.

[117] Casey A, Davoren M, Herzog E, Lyng F M, Byrne H J, Chambers G, Probing the interaction of single walled carbon nanotubes within cell culture medium as a precursor to toxicity testing, Carbon 2007;45: 34–40.

[118] Guo L, Von Dem Bussche A, Buechner M, Yan A, Kane A B, Hurt R H, Adsorption of Essential Micronutrients by Carbon Nanotubes and the Implications for Nanotoxicity Testing. Small 200; 4 (6): 721–727.

[119] Hebert E. Improvement of exogenous DNA nuclear importation by nuclear localization signal-bearing vectors: A promising way for non-viral gene therapy. Biol Cell 2003; 95 (2):59-68.

[120] Wienhues U, Hosokawa K, Hoveler A, Siegmann B, Doerfler W. A novel method for transfection and expression of reconstituted DNA-protein complexes in eukaryotic cells. DNA 1987; 6 (1): 81-9.

[121] Bottger M, Zaitsev S V, Otto A, Haberland A, Vorob´ev V I. Acid nuclear extracts as mediators of gene transfer and expression. Biochim Biophys Acta 1998; 1395 (1):78-87.

[122] Masuda T, Akita H, Harashima H. Evaluation of nuclear transfer and transcription of plasmid DNA condensed with protamine my microinjection: The use of a nuclear transfer score. FEBS Lett 2005; 579 (10): 2143-48.

[123] Morán M C; Vidal L, Fortuño J, Nogueira D R, Vinardell M P. Nuclear proteins on the formation of DNA gel particles as controlled DNA delivery systems . 4th International Congress Nanotechnology, Medicine & Biology (BioNanoMed 2013), Krems,13-15 March 2013.

TABLES

Table 1
Characteristics of the obtained DNA gel particles (see abbreviation list).

Cationic agent	Nucleic acid type		Reference
	dsDNA	**ssDNA**	
SINGLE-TAIL SURFACTANT			
CTAB	x	x	[52, 53]
DTAX	x	x	[54]
ALA, LAM	x	x	[55]
DOUBLE-TAIL SURFACTANT			
DDAB	x	x	[56]
PROTEIN			
LS, PS	x		[52,63]
POLYSACCHARIDE			
CHIT	x		[74]
CL	x		[75]
MIXED SYSTEMS			
LS/PS	x		[63, 64]
CL/PS	x		[75]

Table 2
Mean size of DNA particles systems determined by photon correlation spectroscopy (including polydispersity index, P. I.).

System	Mean size (nm)	P. I.
CTAB-dsDNA	139 ± 8	0.46 ± 0.09
CTAB-ssDNA	126 ± 1	0.40 ± 0.01
PS-dsDNA	387 ± 81	0.78 ± 0.03
PS-ssDNA	343 ± 10	0.14 ± 0.03
LS-dsDNA	9200 ± 2800	0.31 ± 0.40
LS-ssDNA*	9450 ± 2000	0.78 ± 0.50

Table 3

Cytotoxic properties of the protein-based DNA carrier systems prepared by the nebuliser method. Note that the IC_{50} values are given in terms of protein concentration (mM). *Significantly different at $p<0.05$

System	3T3 IC_{50} (mM)	HeLa IC_{50} (mM)
LS-DNA	< 0.16	< 0.16
LSPS15-DNA	> 1.25	0.92
LSPS30-DNA*	> 1.25	0.32
LSPS50-DNA	> 1.25	0.92
LSPS70-DNA	0.80	0.60
LSPS85-DNA*	0.75	0.68
PS-DNA	> 1.25	> 1.25

FIGURE CAPTIONS

Fig. 1. The formation of DNA gel particles: (a) phase map of the CTAB/dsDNA/water mixture a t 25 ºC, where 1Φ and 2Φ indicate the one and two phase regions, respectively and the solid line is the boundary between these regions. (x) Gives studied compositions and (○) the area where gel particles were observed and (b) the representative morphology of CTAB-dsDNA particles. Adapted from reference [52].

Fig.2. Characterization of the DNA gel particles with respect to DNA loading efficiency (a), loading capacity (b) and DNA complexed (c), as a function of the cationic compound (surfactants: light grey bars; proteins: dark grey bars; polysaccharides: black bars). Representative images of the obtained DNA gel particles showing translucent or opaque particles as a consequence of the characteristics of the gelation process (d). Adapted from references [53-56, 63, 74].

Fig. 3. Fluorescence microscopy micrographs of individual surfactant-DNA gel particles in the presence of the DNA selective dye AO (a). Complexed DNA is related to the amounts of DNA in the supernatant solutions and the skins derived from the particles, after particles were magnetically stirred overnight (b) Adapted from references [53-55].

Fig.4. Scanning electron micrographs of individual CTAB–DNA and DTAB-DNA gel particles: outer surface (a) and cross-sections showing both the outer and inner surfaces (b). Adapted from references [52, 56].

Fig. 5. Scanning electron micrographs (a) and fluorescence microscopy using Nile Red (b) and Acridine orange (c) dyes of individual DDAB-dsDNA and DDAB-ssDNA gel particles. Adapted from reference [56].

Fig. 6. Time-dependent changes in relative weight RW measurements performed on surfactant-DNA (a), protein-DNA (b) and polysaccharide-DNA (c) gel particles after exposure to pH 7.6 10 mM Tris HCl buffer solutions. Where W_i stands for the initial weight of the particles and W_t for the weight of the particles at time *t*. Adapted from references [53-56, 63, 74].

Fig. 7. Time-dependent changes in DNA release measurements performed on surfactant-DNA (a), protein-DNA (b) and polysaccharide-DNA (c) gel particles, after exposure to pH 7.6 10 mM Tris HCl buffer solutions. Adapted from references [53-56, 63, 74].

Fig. 8. Adsorption isotherms of Rose Bengal onto CL-DNA gel particles as a function of the R values. (a), onto CL/PS-DNA gel particles as a function of the protein composition (b) and Γ_{max} values of Rose Bengal for these DNA gel particles as a function of the imposed compositions (c). Adapted from reference [75].

Fig. 9. Dependence of rat erythrocyte haemolysis on DTA-based surfactant concentration. Erythrocytes were incubated for 10 min at room temperature at different surfactant concentrations, and the amount of haemoglobin released was determined (a). DTA-dsDNA (b) and DTA-ssDNA particle-induced haemoglobin release from rat erythrocytes as a function of time(c). Erythrocytes were incubated at room temperature in the presence of individual DTA-DNA particles. The data correspond to the average of three independent experiments ± standard deviation. Adapted from reference [54].

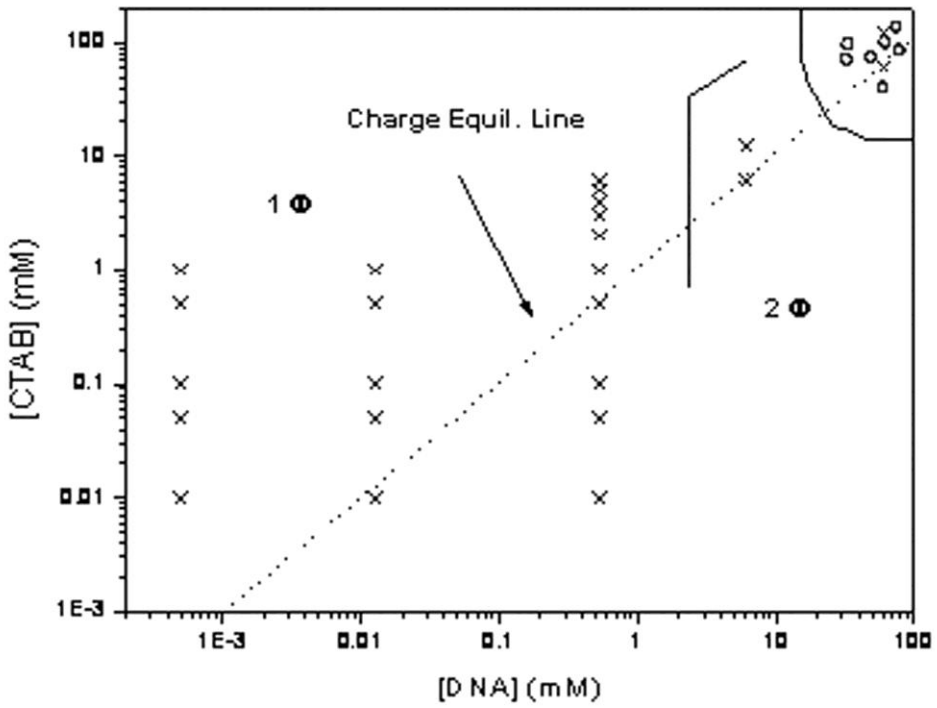
Fig. 10. Relative kinetics of DTA-dsDNA (a) and DTA-ssDNA (b) particle-induced haemoglobin release from rat erythrocytes and DNA release. Adapted from reference [54].

Fig. 11. Concentration-dependent relative viabilities of 3T3 cells (A) and HeLa cells (B) treated with LS and PS for 24 h determined by MTT assay. The data correspond to the average of three independent experiments ± standard deviation. Adapted from reference [64].

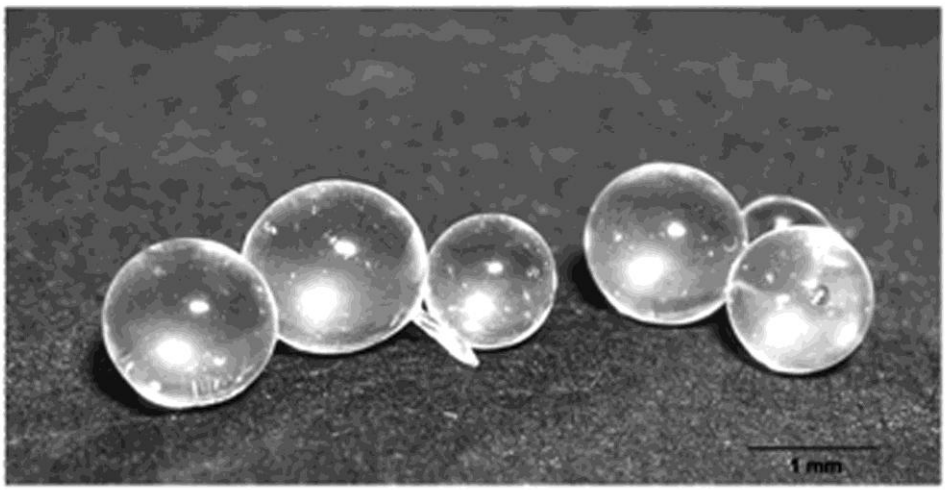
Fig. 12. Relative viabilities of 3T3 and HeLa cells treated with individual protein-DNA gel particles (a) and the corresponding protein solutions derived from the dropwise addition method (b) for 24 h, determined by MTT assay. The data correspond to the average of three independent experiments ± standard deviation. *Significantly different ($p<0.05$) from the corresponding protein solution. Adapted from reference [64].

Fig. 13. Relative viabilities of 3T3 and HeLa cell lines treated with the protein-DNA gel particles dispersion (a) and the corresponding protein solutions derived from the nebulisation method (b) for 24 h, determined by MTT assay. In both cases, the assayed concentration was 1.25 mM, expressed in terms of protein concentration. The data correspond to the average of three independent experiments ± standard deviation. * Significantly different ($p<0.05$) from the corresponding protein solution. Complexation stoichiometries of the protein-DNA gel particles according to the protein composition(c). Comparison between the complexation stoichiometries of the protein-DNA gel particles and the relarive viabilities of 3T3 and HeLa cell lines according to protein composition. Adapted from reference [64].

Fig. 14. Concentration-dependent relative viabilities of 3T3 (A) and HeLa cells (B) treated with the protein-DNA gel particles prepared by the nebulisation method for 24 h, determined by MTT assay. The data correspond to the average of three independent experiments ± standard deviation. Adapted from reference [64].



a)



b)

Figure 1

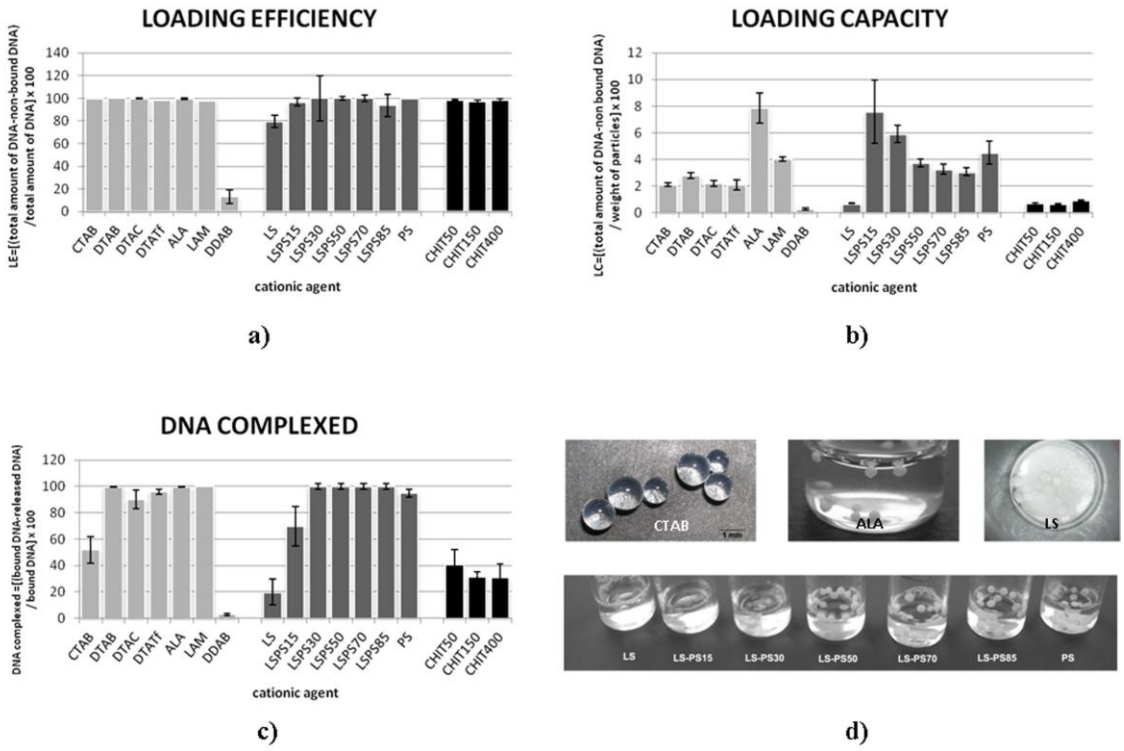
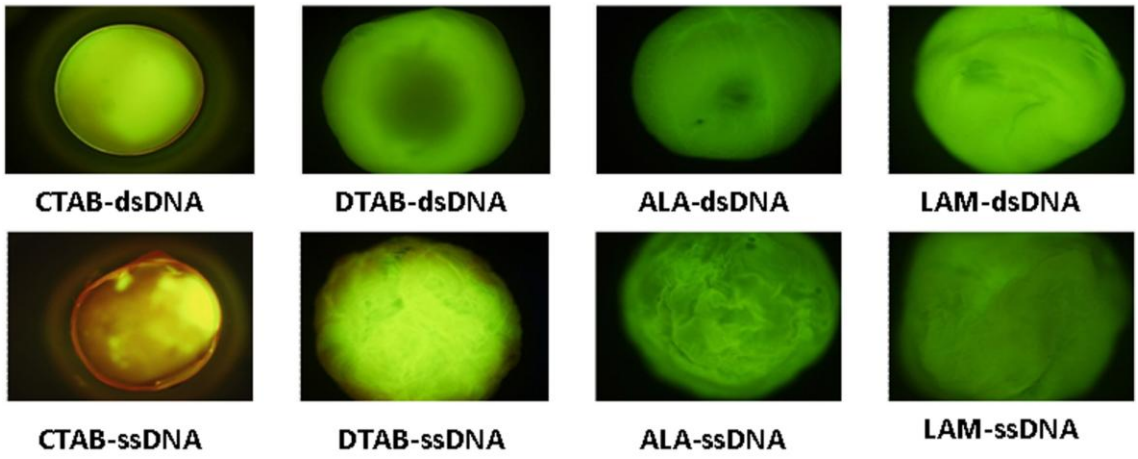
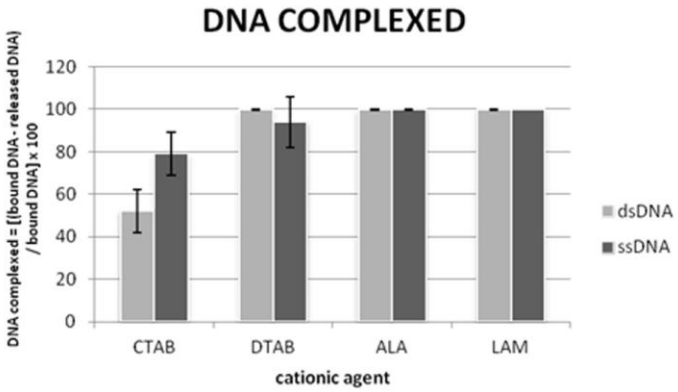


Figure 2



a)



b)

Figure 3

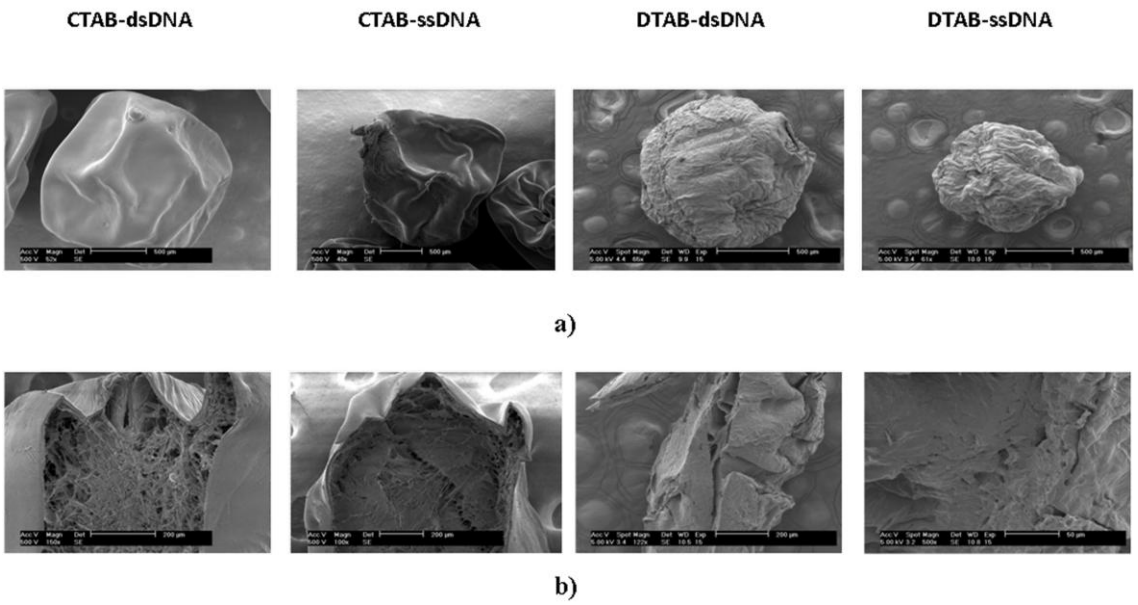


Figure 4

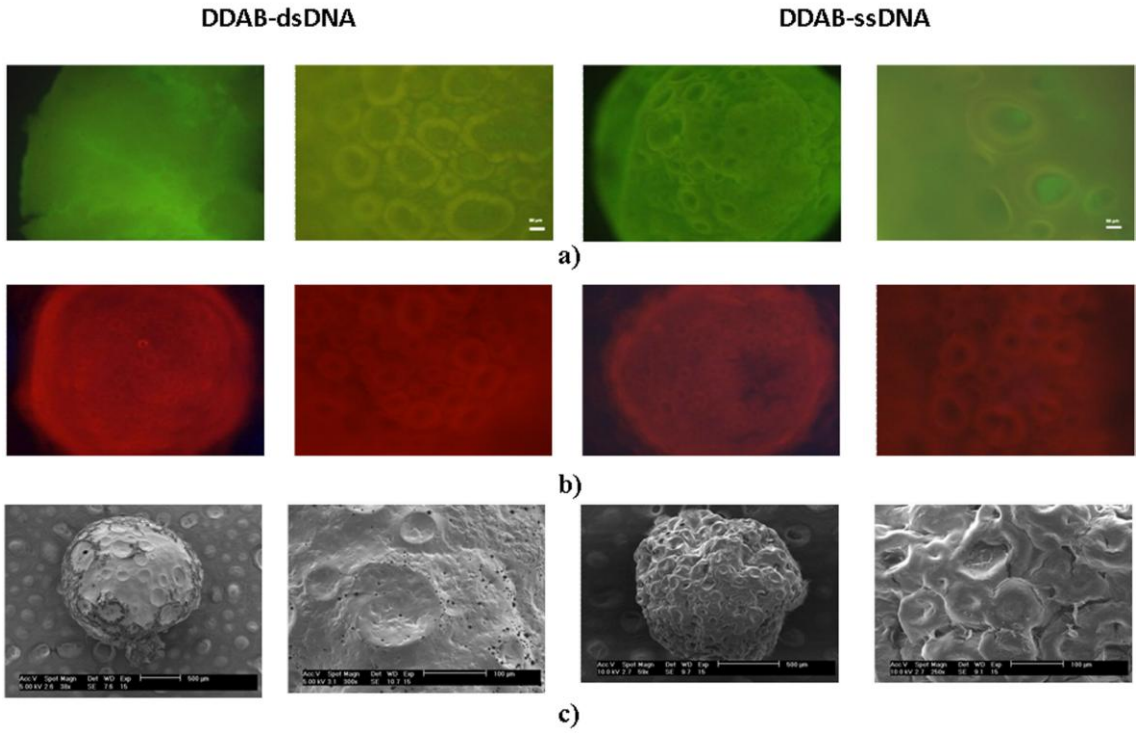


Figure 5

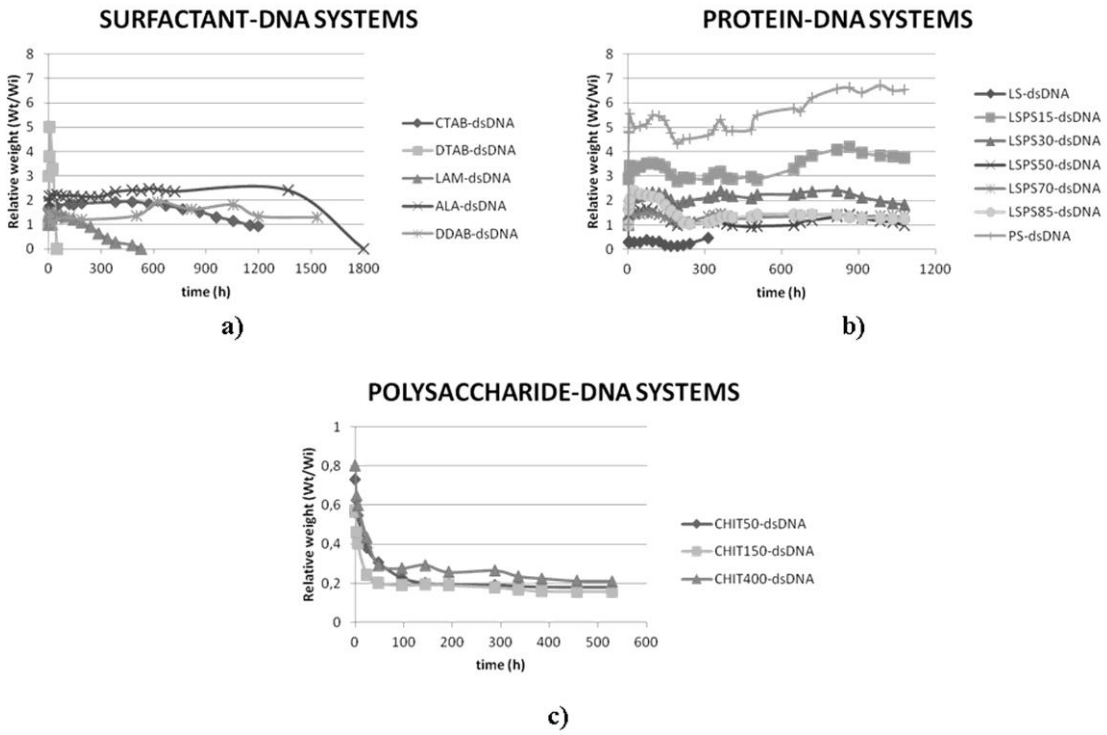


Figure 6

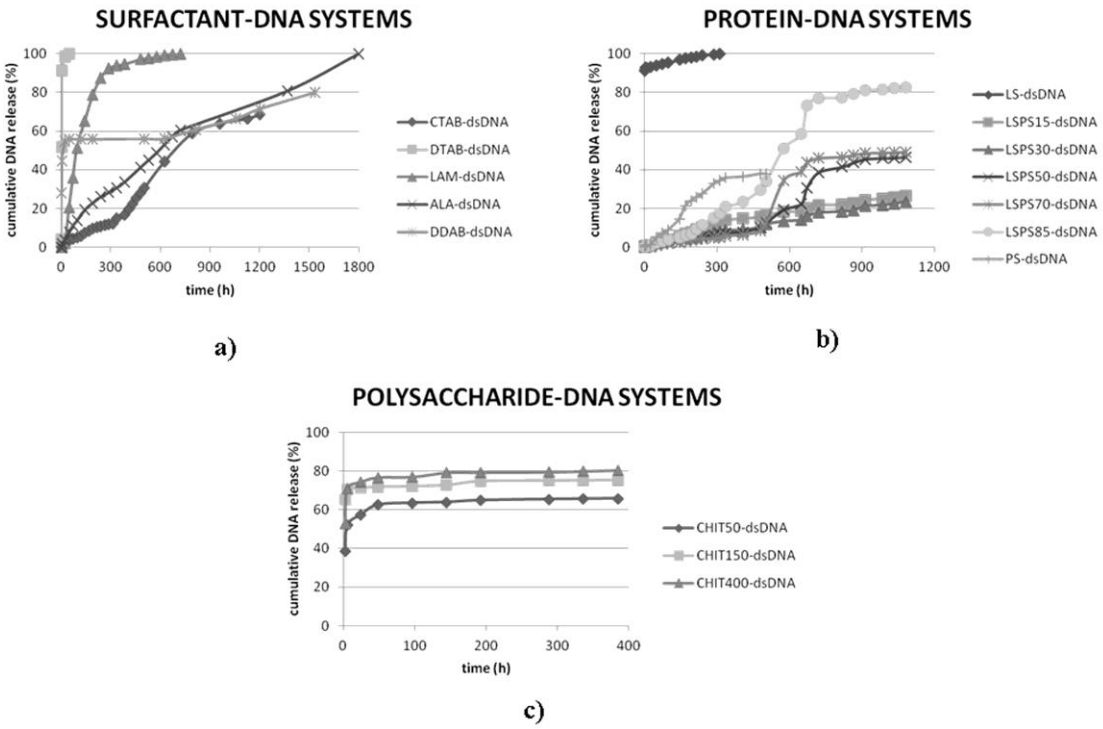


Figure 7

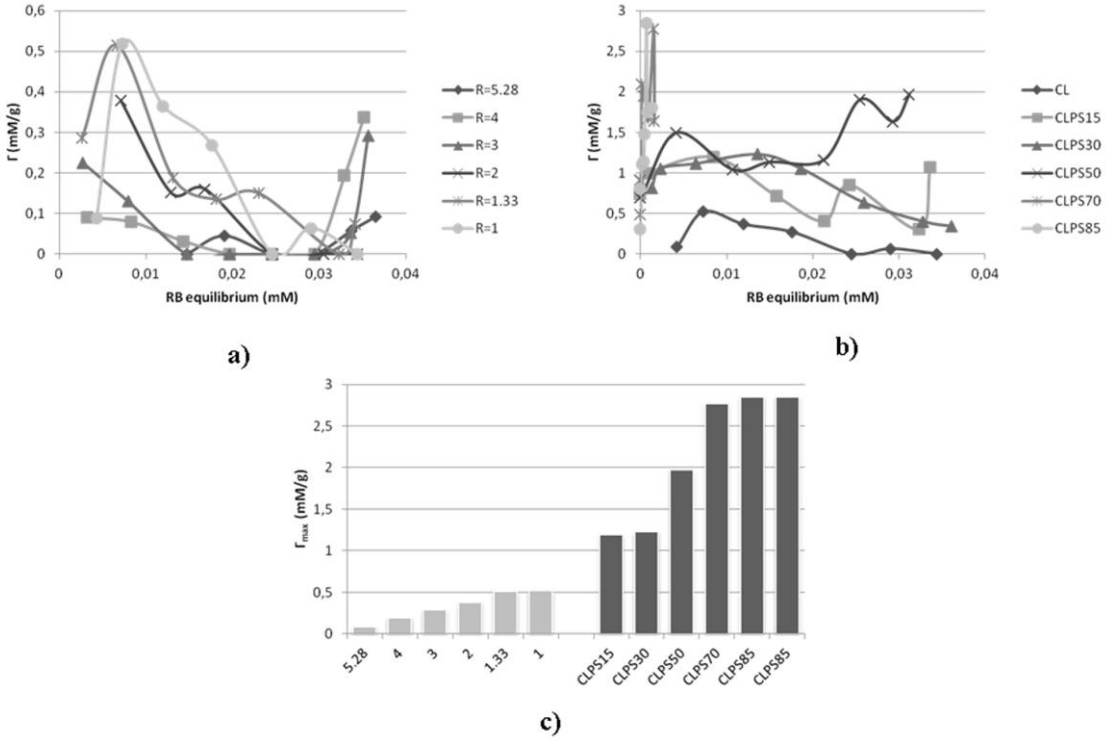


Figure 8

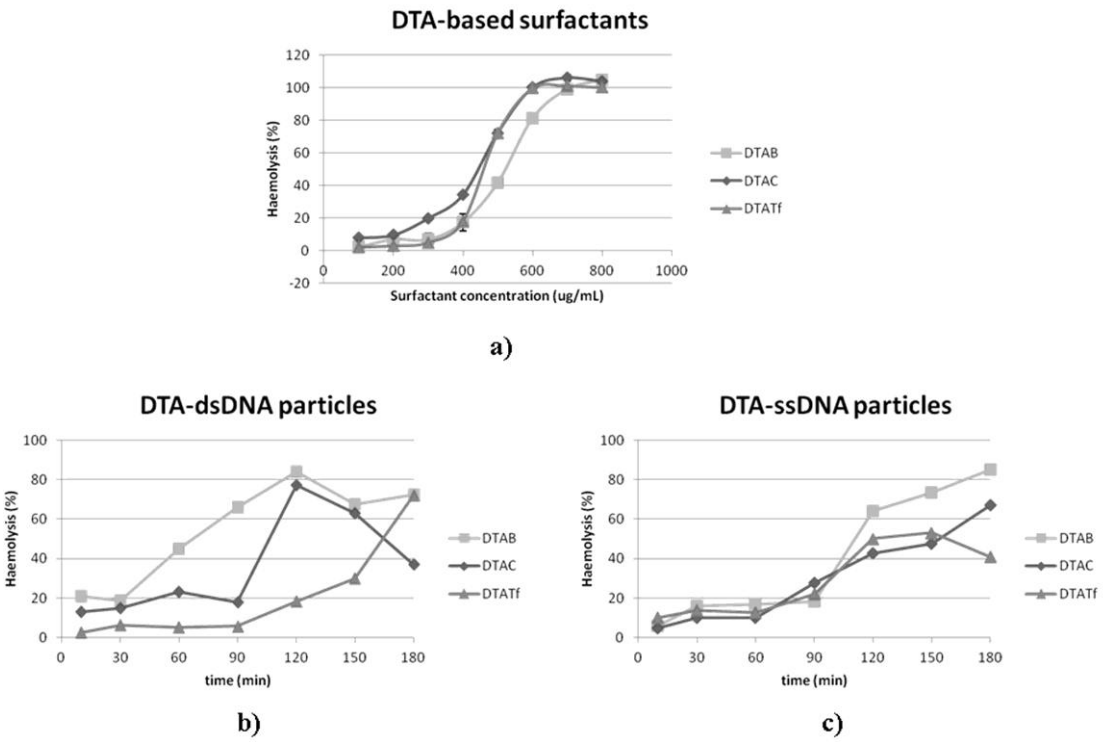


Figure 9

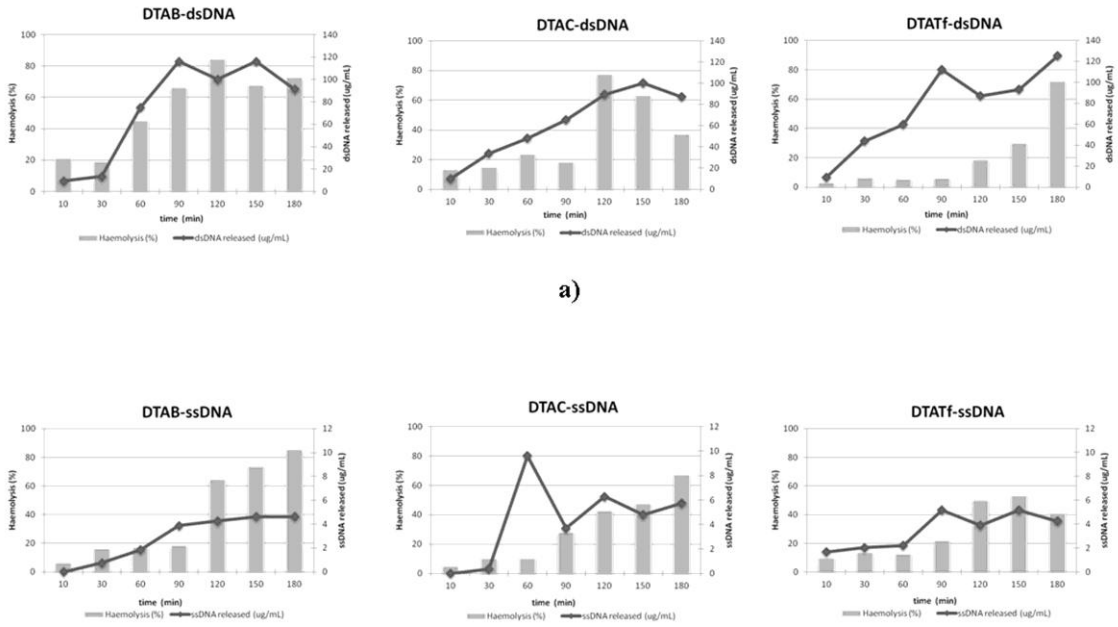


Figure 10

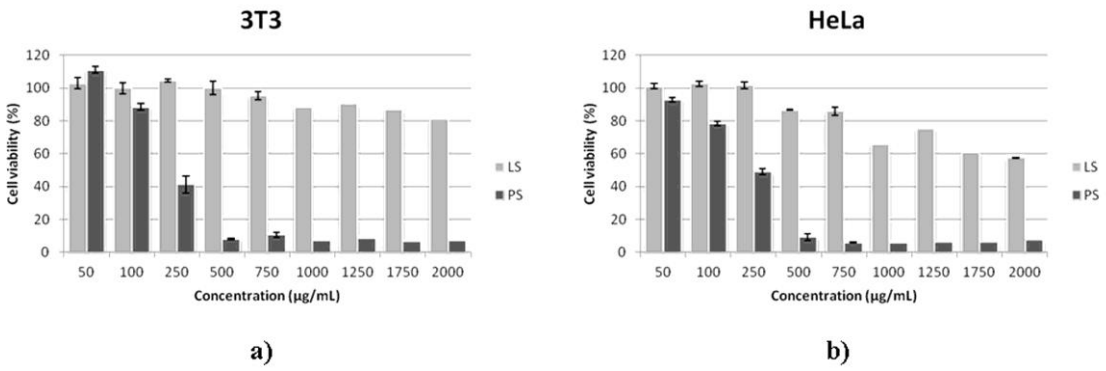


Figure 11

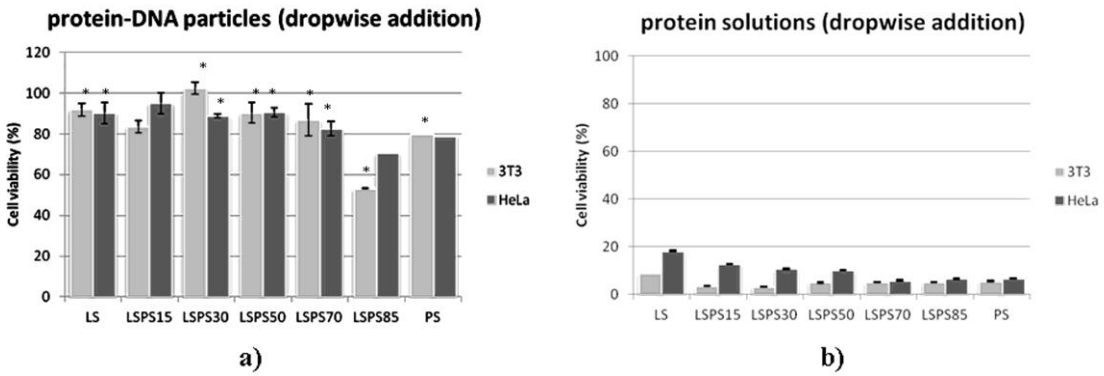


Figure 12

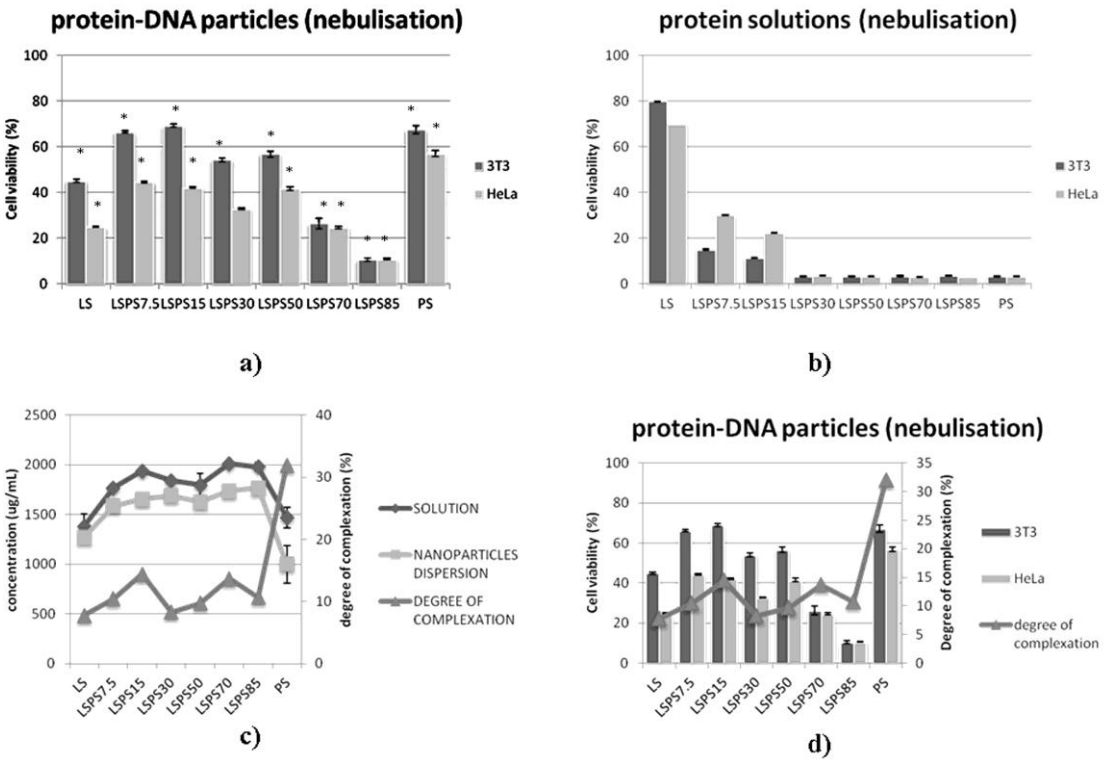
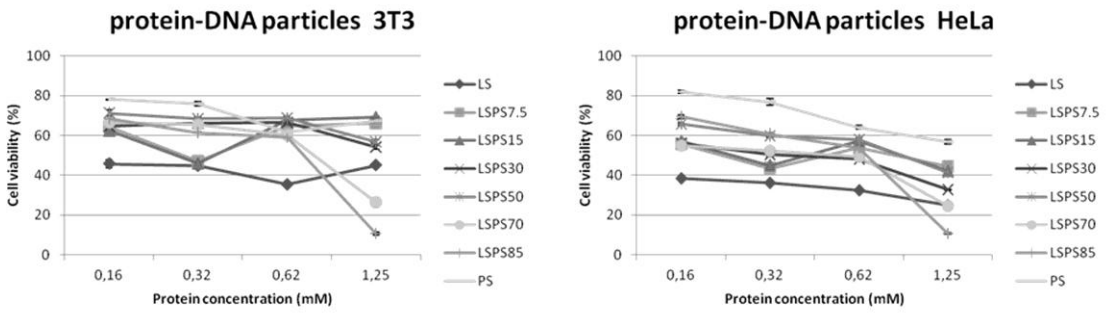
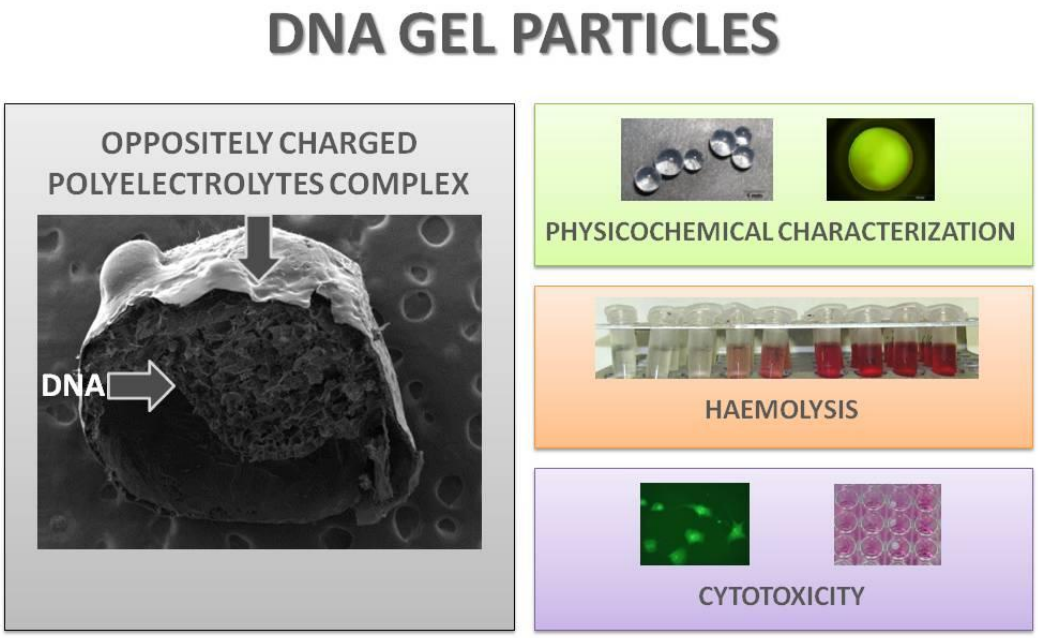


Figure 13



a) **b)**

Figure 14



Graphical abstract

Highlights

►Comprehensive review of DNA gel particles as controlled DNA delivery systems. ► DNA gel particles derived from surfactants, proteins and polysaccharides derivatives ► Kinetics of swelling or deswelling, dissolution, and DNA release. ► *In vitro* haemolytic and cytotoxic characterization ► Current status and prospects on DNA gel particles are discussed.