



Transworld Research Network  
37/661 (2), Fort P.O.  
Trivandrum-695 023  
Kerala, India

Recent Advances in Pharmaceutical Sciences III, 2013: 145-162 ISBN: 978-81-7895-605-3  
Editors: Diego Muñoz-Torrero, Amparo Cortés and Eduardo L. Mariño

## 9. Current directions in DNA gel particles

M. Carmen Morán<sup>1,2</sup>, Montserrat Mitjans<sup>1,2</sup>, Verónica Martínez<sup>1</sup>  
Daniele R. Nogueira<sup>1</sup> and M. Pilar Vinardell<sup>1,2</sup>

<sup>1</sup>*Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona  
Avda. Joan XXIII, 08028 Barcelona, Spain;* <sup>2</sup>*Interaction of Surfactants with Cell Membranes  
Unit Associated with CSIC, Facultat de Farmàcia, Universitat de Barcelona  
Avda. Joan XXIII, 08028 Barcelona, Spain*

**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) DNA. This paper reviews the preparation of DNA gel particles using surfactants, proteins and polysaccharides. Particle morphology, swelling/dissolution behaviour, degree of DNA entrapment and DNA release responses as a function of the nature of the cationic agent used are discussed. Current directions in the haemocompatible and cytotoxic characterization of these DNA gel particles have been also included.

### Abbreviations

ALA : arginine-N-lauroyl amide dihydrochloride  
CHIT : chitosan

Correspondence/Reprint request: Dr. M. Carmen Morán, Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Avda. Joan XXIII, 08028 Barcelona, Spain. E-mail: mcmoranb@ub.edu

CTAB	: cetyltrimethylammonium bromide
DDAB	: didodecyldimethylammonium bromide
DTAB	: dodecyltrimethylammonium bromide
DTAC	: dodecyltrimethylammonium chloride
DTATf	: dodecyltrimethylammonium trifluoromethane sulfonate
LAM	: N <sup>α</sup> -lauroyl-arginine-methyl ester hydrochloride
LS	: lysozyme
MTT	: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PS	: protamine sulfate

## Introduction

The idea of 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. A number of strategies are applied to limit DNA degradation, such as complexation with cationic species. DNA is a highly charged polyelectrolyte and therefore associates strongly with any oppositely charged cosolute, including simple ions, polymers, proteins, surfactants, lipids and other bioparticles. The interactions of cationic cosolutes with DNA have been extensively studied [2]. In general, strong associative phase separation is observed. The driving force for this strong association is electrostatic interaction between the two components. This is given by entropic increase due to the release of the respective counter ions.

A general understanding of the interactions between DNA and oppositely charged agents, and in particular the phase behaviour, has provided the basis for developing novel DNA-based materials, including gels, membranes and gel particles [2-4]. We have recently prepared novel DNA gel particles based on associative phase separation and interfacial diffusion. The polyelectrolyte of interest, DNA, is located in the core of these particles, while the complex between oppositely charged polyelectrolytes forms the corresponding shell. By mixing solutions of either single- (ssDNA) or double-stranded (dsDNA) DNA with solutions of different cationic agents, such as surfactants, proteins and polysaccharides, the possibility of forming DNA gel particles, without adding any kind of cross-linker or organic solvent, has been confirmed. Table 1 summarizes the characteristics of the different DNA gel particle systems. The association strength, which is altered by varying the chemical structure of the

**Table 1.** Characteristics of the obtained DNA gel particles (see abbreviation list at the end of the chapter).

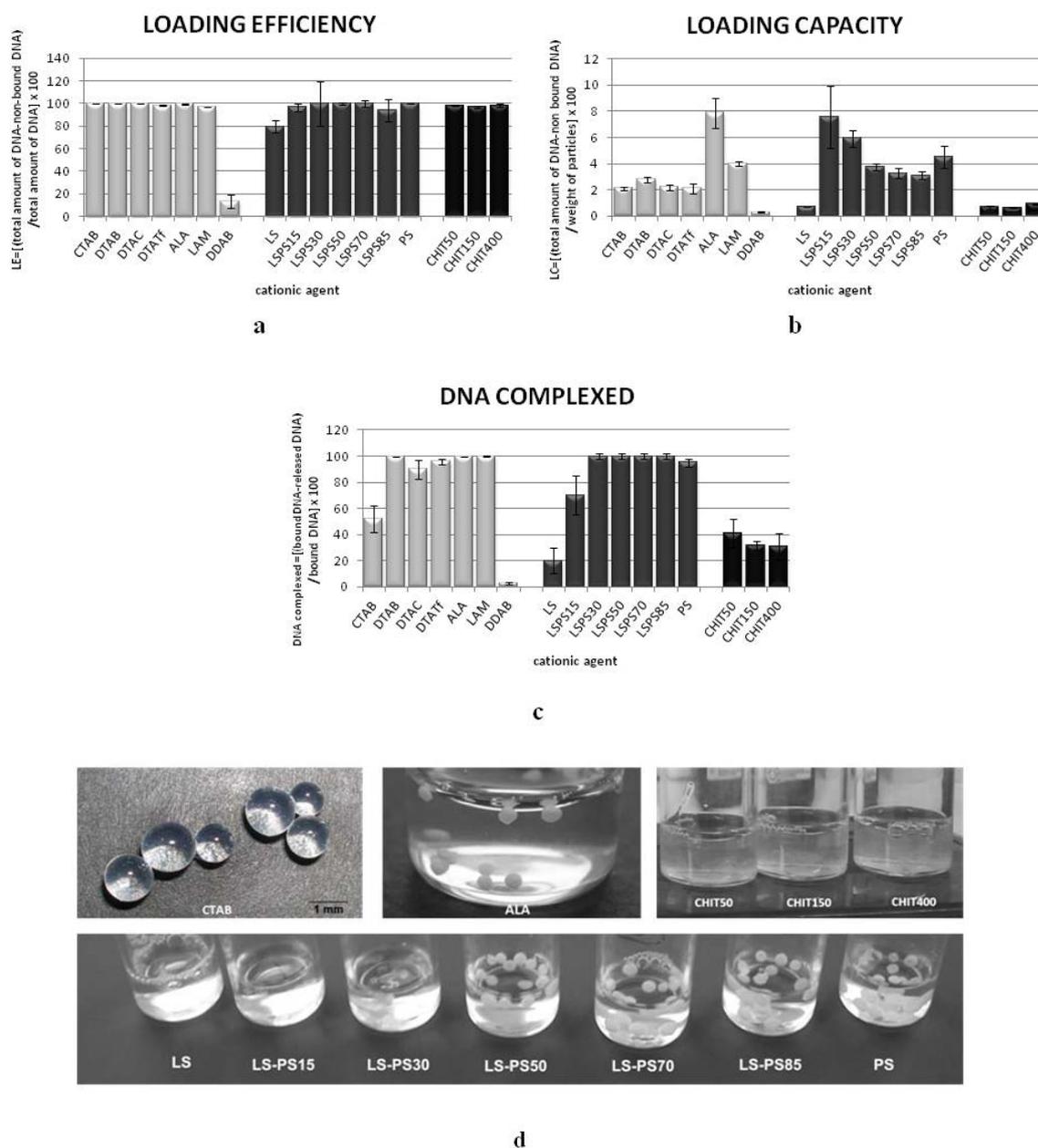
CATIONIC AGENT	dsDNA	ssDNA	Reference
<b>SINGLE-TAIL SURFACTANTS</b>			
CTAB	x	x	[5,6]
DTAX	x	x	[7]
ALA, LAM	x	x	[8]
<b>DOUBLE-TAIL SURFACTANTS</b>			
DDAB	x	x	[9]
<b>PROTEINS</b>			
LS, PS	x		[5, 10]
<b>POLYSACCHARIDES</b>			
CHIT	x		[11]
<b>REVIEW</b>			[12]

cationic agent, enables the spatial homogeneity of the gelation process to be controlled to produce either a homogeneous DNA matrix or different DNA reservoir devices. These can represent a “bridge” for potential applications in the controlled encapsulation and release of ssDNA and dsDNA, with clear-cut differences in the mechanism. 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.

## 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 DNA gel particles, which are formed using surfactants, proteins, and polysaccharides as cationic compounds, are summarized in Figure 1. 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 these cationic solutions (Fig. 1a). However, the entrapped



**Figure 1.** 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 [5, 8,10].

DNA, as a function of the weight of the particles (LC values), depends on the cationic compound used (Fig 1b). The LC values obtained for surfactant-DNA gel particles depends on the hydrophilic and hydrophobic contributions. For the same type of polar head (CTAB and DTAB structures), the

hydrophobic contribution did not have a strong influence on the observed LC value [6, 7]. Identical LC values were obtained when DNA gel particles were prepared with surfactants that only differ in counter ion structure (DTAB, DTAC, DTATf) [7]. However, for the same hydrophobic chain length (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 [8]. When the DTAB and DDAB structures are compared, we can conclude that higher number of hydrophobic chains on the surfactant structure contributes negatively to the LC values [9]. In the case of protein-DNA gel particles, the lowest LC value (0.7%) was obtained in particles formed with pure LS. Interestingly, highest LC value was obtained for particles containing the smallest amount of PS [10]. Molecular weight of the polysaccharide structure did not have a clear effect on the LC values [11].

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. 1c. 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, when surfactant structures are used with twelve carbon atoms in the hydrophobic chain (DTAB, DTAC, DTATf, ALA, LAM), most of the DNA is complexed during particle formation [7, 8]. More limited complexation has been obtained by increasing, either the alkyl chain length (CTAB), or the number of alkyl chains in the molecule (DDAB) [6, 9]. In the case of the protein-DNA gel particles, the amount of complexed DNA increases progressively in the presence of the protein protamine sulphate [10]. 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 [11].

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 [13]. 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. 1d).

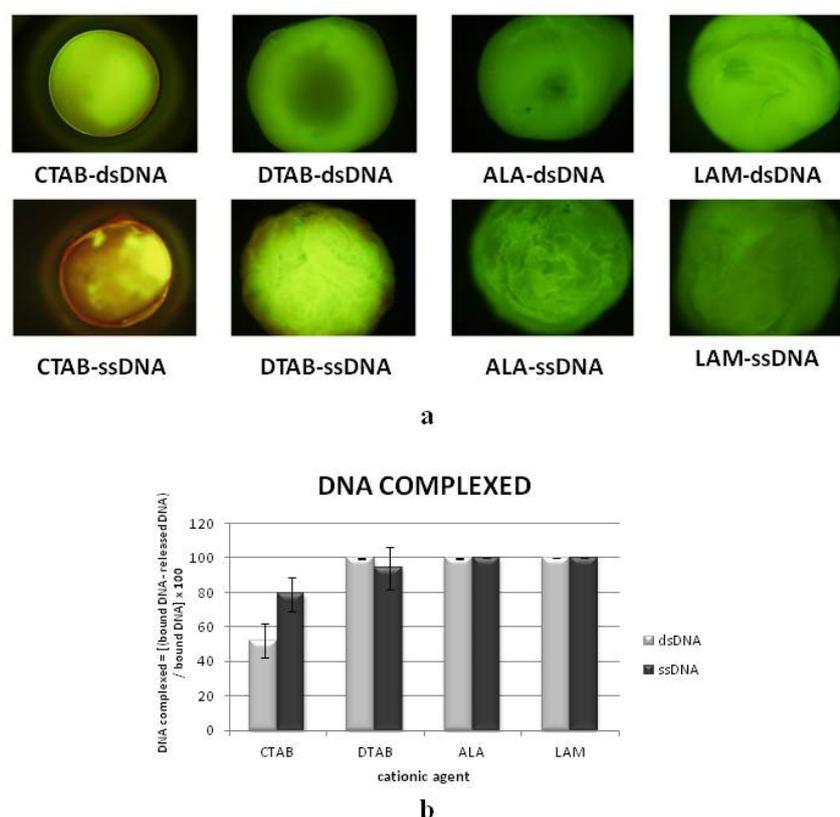
## 2. Morphological characterization of the DNA gel particles

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 [14-17]. 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 DNA, and denatured, single-stranded DNA, in the gel particles. Fig. 2a shows fluorescence micrographs of individual particles of the surfactant-dsDNA systems.

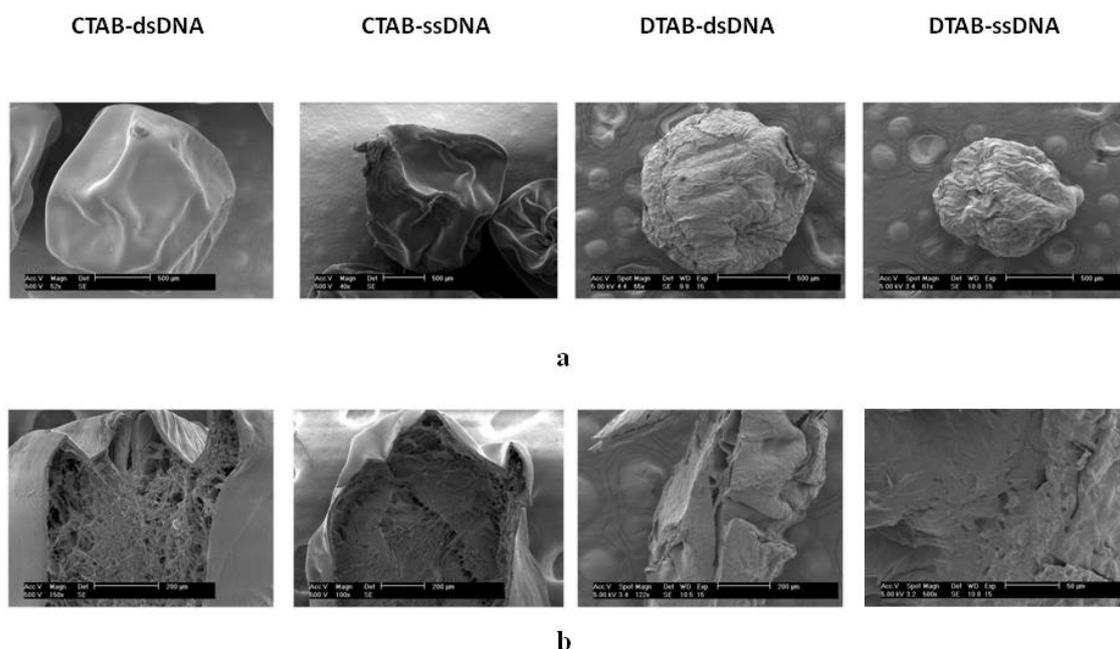
FM studies have revealed that the formation of particles with double-stranded DNA is carried out with conservation of the secondary structure of the DNA. However, in the case of particles formed with denatured DNA, green emission is also observed, except in the case of CTAB-ssDNA gel particles. The absence 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. 2b). 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.



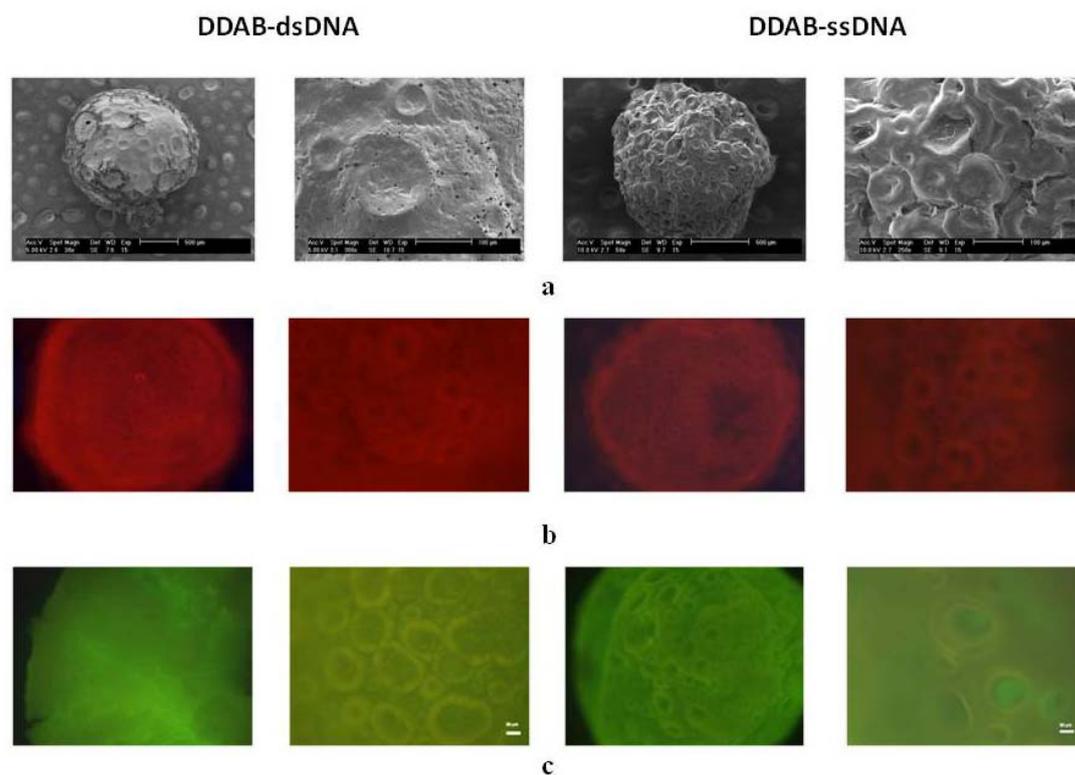
**Figure 2.** Fluorescence microscopy micrographs of individual surfactant-DNA gel particles in the presence of the DNA selective dye AO (a). Complexed DNA is related to the amounts of DNA in the supernatant solutions and the skins derived from the particles, after particles were magnetically stirred overnight (b) Adapted from references [6,8,9].

Scanning electron microscopy imaging was carried out to establish possible differences in the morphologies between the different particles. Figure 3 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. 2b), which increases the shell section of the obtained particles.

Similar experiments were carried out on particles formed with the double-tail surfactant DDAB [9]. In this case, spherical domains formed that were visible on the surface of these DDAB-DNA particles (Fig. 4a). The nature of these domains was studied using the hydrophobic dye Nile Red (NR) (Fig. 4b) [18]. The fluorescence emission of NR, in the presence of DDAB-DNA gel particles, was nearly identical to that recorded for DDAB vesicles. This indicates that the observed spherical domains on the surface of the DDAB-DNA gel particles are composed of hydrophobic layers of the surfactant. The examination of the DDAB-DNA gel particles with the DNA-selective dye AO revealed that the DNA is also included in these domains. Differences in the reorganization of DNA were found as a function of the secondary structure.



**Figure 3.** 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 [5,9].



**Figure 4.** 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 [9].

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.

### 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 the erosion of them. When the DNA gel particles are inserted into a certain medium, different responses occur: swelling or deswelling, dissolution, and release of DNA.

In the case of surfactant-DNA gel particles, the extension of the swelling process depends on both the surfactant and the nucleic acid structures (Fig 5a). For the same hydrophilic contribution (CTAB and DTAB), the decrease in the number of carbon atoms in the hydrophobic chain contributes negatively to the swelling extension. 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) [6]. However, DTAB-DNA particles showed an initial swelling and then dissolved completely after 48 h. DTAB-DNA gel particles have the largest relative weight (RW=4–6, depending on the secondary structure of the nucleic acid) [7].

Generally, the release pattern resembles that observed in the swelling/dissolution profiles (Fig. 6a). Thus, CTAB-DNA particles placed in pH 7.6 10 mM Tris HCl buffer showed no initial burst release: in the first 24 h, only 1.6, 2.0 and 3.3% of DNA was released, respectively. After 1,200 h, 69 and 40% of DNA was released from the particles containing dsDNA and ssDNA, respectively [6]. Nevertheless, DTAB-DNA particles exhibited fast release behaviour by a dissolution mechanism. The corresponding half-lives of DTAB-dsDNA and DTAB-ssDNA are 4 and 8 h, respectively. After 24 h, more than 97% of the bound DNA was released [7].

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 [8]. Particles containing ALA exhibited the largest (relative weight ratio, RW 2-2.5) and the longest (more than 1,300 h) swelling process. Particles containing LAM swelled (RW 1.5-2.0 using the maximum points as estimate) for up to 10 or 200 h, as a function of the secondary structure of the polyelectrolyte, and then started to shrink. The results suggest that the stability of the gel particles is given mainly by the electrostatic interaction between DNA and the oppositely charged surfactant. More stable particles were obtained for ALA than for LAM, probably due to its double charge. In addition, in the latter case, the stability was higher when denatured DNA was used. Thus, LAM-DNA particles exhibited faster release than ALA-DNA particles (Fig. 6a). Complete release from LAM-dsDNA particles occurred after 400 h; whereas complete DNA release from LAM-ssDNA occurred after 800 h. When the formulation contained ALA, the DNA release was slower. Complete DNA release was only achieved after 1800 h. With respect to the observed differences between ssDNA and dsDNA release, the results agree with our previous studies on surfactant and protein systems, which showed a stronger interaction with ssDNA than with dsDNA [5,6,]. The rate and the final cumulative DNA release depend on its secondary structure. This strongly

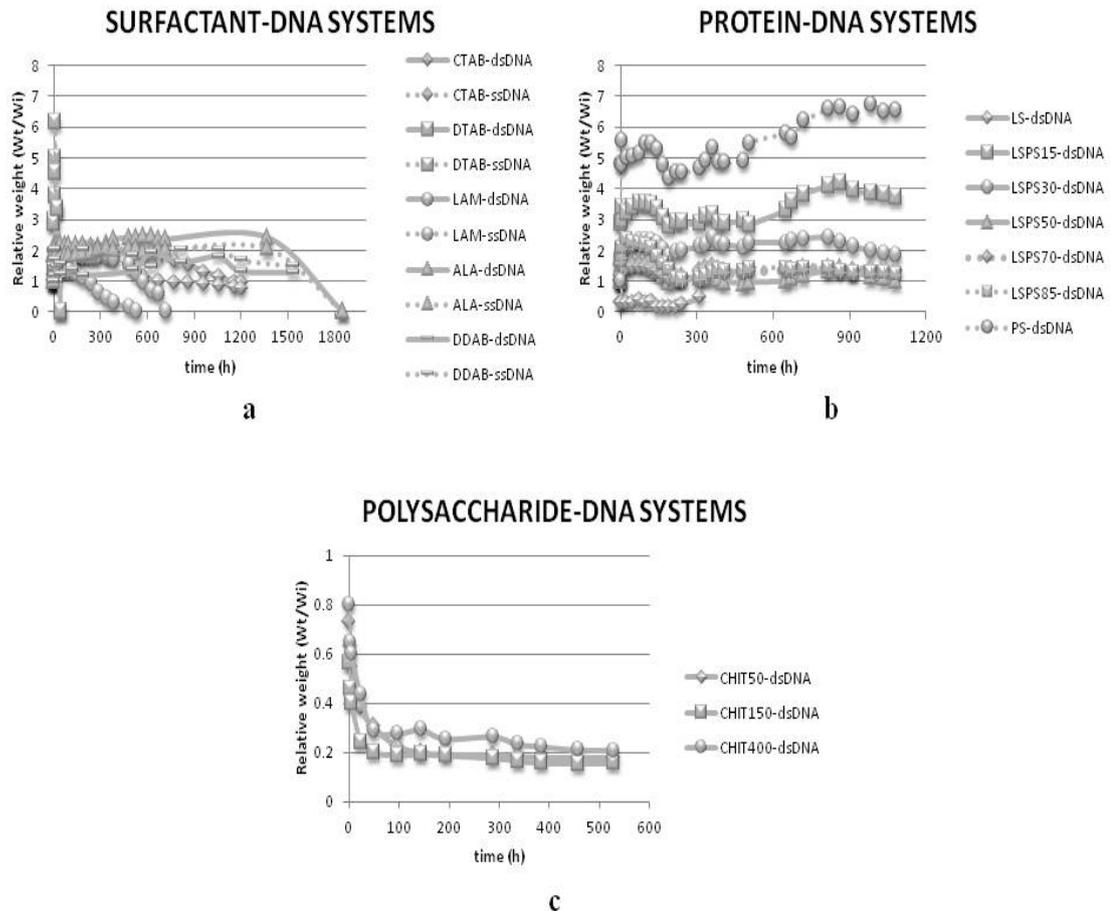
indicates the important role of hydrophobic interaction in DNA when the bases are more exposed, as in the case of ssDNA.

Furthermore, the increase in the number of alkyl chains (DDAB) contributes positively to the stabilization of the particles [9]. 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 6a). The duration of this burst release (24 h) was independent of the secondary structure of the nucleic acid used, but not of the amount released. The amount of DNA released in the first 24 h was 44% and 15% for DDAB-dsDNA and DDAB-ssDNA particles, respectively. 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 and DDAB-ssDNA was 63 and 34%, respectively.

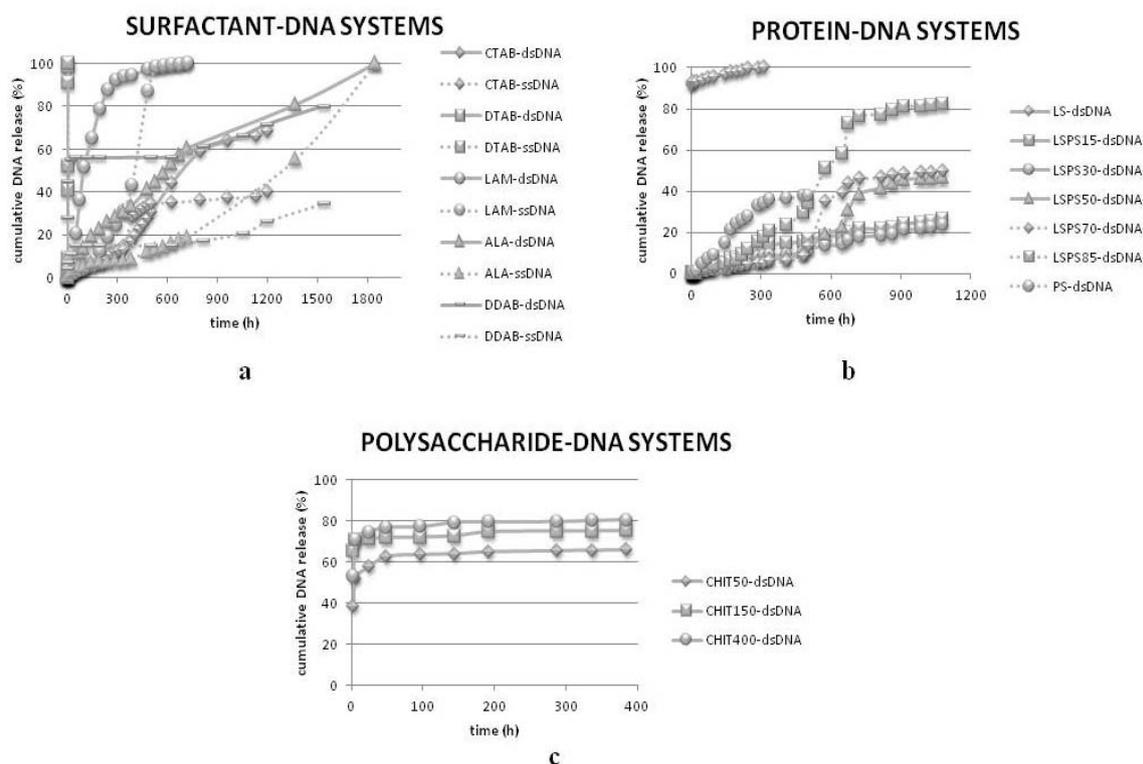
Concerning protein-DNA gel particles, LS-DNA gel particles lost weight rapidly and extensively (Fig. 5b) [10]. In the case of PS-DNA particles, the largest relative weight ratio was observed ( $RW > 5$ ). For the high LS/PS ratio, particles absorbed a water amount of 2-3 times the initial mass (relative 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-DNA particles, RW increased with time, while for the LS-PS15 system, nonmonotonic behaviour was observed.

LS-DNA particles exhibited fast burst release behaviour by a dissolution mechanism (Fig. 6b). 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  $\approx 400$  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. 5b). 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 Figure 5, 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.



**Figure 5.** 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 [10,11].



**Figure 6.** 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 [10,11].

The determination of the kinetics of swelling and dissolution behaviour demonstrated that chitosan-DNA gel particles lost weight rapidly and extensively (Fig. 5c). 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. 6c. 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, medium and high molecular weight chitosan, respectively [11].

#### 4. Determination of *in vitro* biocompatibility

The safety evaluation of new products or ingredients destined for human use is crucial prior to exposure. Rapid, sensitive and reliable bioassays are required to examine the toxicity of these substances. Established cell lines are useful alternative test systems for this kind of toxicological studies [19]. However, they must be chosen with care with regard to their origin [20]. Cytotoxicity assays are among the most common *in vitro* endpoints used to predict the potential toxicity of a substance in a cell culture [21]. It is

essential to understand the interactions of the DNA-based particles with cells *in vitro* to improve their behaviour *in vivo*.

Nano-sized materials (NMs) have a high potential in technical and medical applications provided they are not toxic. Despite the significant scientific interest and promising potential, the safety of nanoparticulate systems remains a growing concern, considering that biological applications of nanoparticles could lead to unpredictable effects. 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. These screening assays are also used for NMs. As a general rule, nano-sized materials show higher reactivity than bulk materials of the same composition. Therefore, toxicity data must be interpreted in the context of the physicochemical characteristics of the NM [22].

Size, surface charge, and hydrophobicity interact in complex ways and have a pronounced influence on biocompatibility. Aggregation of NMs in physiological fluids is often observed. 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 NMs. Most examples have been published for the MTT assay, which is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide by cellular dehydrogenases. NMs interfere with the assay by light absorbance, reduction of the tetrazolium salt, and binding of the formazan salt [23]. Interactions of NMs with assays include interference by adsorption of dyes [24], absorbance [25], fluorescence [26], binding of proteins [27], dye degradation [28], and dye formation [24] among others. The most important conclusion drawn from these studies was that more than one cytotoxicity assay should be used to evaluate NMs.

No specific cytotoxicity screening assays and no specific cell lines are fixed to study the effect of NMs. Therefore, a variety of cell lines are being used for cytotoxicity screening. Usually, the concentration that has a half-maximal effect on the decrease of viability is considered as the toxic concentration ( $TC_{50}$ ), effective concentration ( $EC_{50}$ ), or inhibitory concentration ( $IC_{50}$ ).

Although no prominent variation between species have been reported for conventional chemicals, NMs may react differently. An investigation of the cytotoxicity of cationic polystyrene nanospheres in five cell lines was reported. It was found that despite uptake in all cell lines, the nanospheres only affected cytotoxicity in two lines [29]. The different reaction of the cell lines may be due to their specific characteristics. Potential parameters that

could influence the results of the cytotoxicity screening of NMs include the phagocytic capacity of the cells. Other cellular parameters, like doubling time, size, embryonic origin etc., could also play their role. Knowledge about such potential differences can help in the design of cytotoxicity testing of new NMs and in explaining cytotoxicity data that varies between laboratories.

One of the most common non-epithelial cell lines used in short- and long-term nanotoxicological *in vitro* studies on: cytotoxicity, biocompatibility, or mechanisms of cellular uptake of nanoparticles, are the 3T3 fibroblasts. These are readily available, undergo contact inhibition, and are more closely representative of a physiologic model than cancer cell lines, for example [30]. Our group studies have demonstrated that the cytotoxicity of cationic nanovesicles differed depending on the cell lines as well as the *in vitro* endpoint that was measured. This shows that the selected cell type and assay can affect the final outcome, as indicated by other authors [22, 24]. Overall, our findings showed that nanovesicle composition plays a primary role in underlying toxicity. The cytotoxic responses of the nanovesicles varied especially as a function of the cationic charge position on the amphiphile that was included in them. Furthermore, the surfactant with the highest hydrophobicity tends to enhance the toxic potential of the formulations. All these findings suggest that differential toxicity according to vesicle composition could be an important concept when new nanomaterials are developed for biomedical applications. In conclusion, the combination of all assays used in the present study offers an in-depth and comprehensive evaluation of the potentially toxic effects of nanomaterials [31].

Another problem involved in the determination of the cytotoxic effect of nanomaterials is interference with the medium and possible changes in size, when they are incorporated into the cell culture medium. The adequate size characterization of nanomaterials is a prerequisite for meaningful outcomes of nanotoxicity studies [32]. Indeed, in contrast to synthetically produced nanoparticles, nanovesicles are much less homogeneous in size and composition. Thus, aggregation of nanovesicles and size distribution will affect the outcome of toxicity tests dramatically, as larger particles may settle faster in cell culture, and are less available to cells than smaller ones, that remain in suspension longer and are thus available for incorporation via endocytosis [33]. Obviously, there are numerous methods for the characterization of nanomaterials [34], but no single method will permit a description of nanovesicle characteristics that can support an improved interpretation of the *in vitro* effect data. In studies of the toxicity of nanovesicles, cytotoxicity assays should be performed for the nanovesicles and for different components such as the cationic surfactants involved in

these particles. Our group has evaluated the cytotoxicity of different cationic surfactants [35,36].

Another important feature in the development of nanoparticulate 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 [7, 37-39]. 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.

Studies of our group carried out with surfactant-DNA gel particles have demonstrated that the amount of DNA that is released and the haemolytic response induced by surfactant-DNA gel particles are strongly dependent on both the structure of the counter ion in the surfactant and the secondary structure of the DNA [7]. One drawback of these surfactant-DNA gel particles, in toxicological terms, is the need for a cationic surfactant, which may cause some cellular damage. However, our results indicate that the effect of the surfactant can be modulated when administered in the DNA system, unlike an aqueous solution. This modulation is due to the strong interaction between the surfactant and the biopolymer, which leads to very slow release of the surfactant from the vehicle.

The surfactant-DNA interaction reflects both the release of haemoglobin (degree of haemolysis) and the release of DNA into the media, as a consequence of the dissolution kinetics of the polyelectrolyte-surfactant complexes. Under the experimental conditions in which the haemolysis studies took place, the amount of dsDNA that is released at the end of the experiment (180 min) reaches 100 mg/mL. However, with particles prepared with denatured DNA, only 10% of this amount is released into the media. These differences are 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. With respect to the surfactant structure, 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.

## 5. Conclusion

A general understanding of interactions between DNA and oppositely charged agents provides a basis for developing novel DNA gel particles. When the DNA gel particles are inserted in a medium, different responses occur: swelling or deswelling, dissolution, and DNA release. 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. While toxicity certainly applies for most classical surfactants, we are engaged in work on haemocompatible and cytotoxic assessments of DNA gel particles prepared with cationic compounds with much improved intrinsic biocompatibility. These include surfactants with the cationic functionality based on an amino acid [8], polysaccharides [11] and proteins [10]. There is special interest in decreasing the size of these DNA gel particles [40], which is a prerequisite for cellular uptake and internalization, and subsequent DNA delivery and transfection.

## Acknowledgements

M.C. Morán acknowledges the support of the MICINN (Ramon y Cajal contract RyC 2009-04683). The authors wish to express their appreciation to Dr. Björn Lindman and Dr. Maria da Graça Miguel from University of Coimbra for their support over the last years. The authors are grateful to Dr. Maria Rosa Infante for helpful suggestions to a previous version of the chapter. This research was supported by the Project MAT2012-38047-C02-01 from the Spanish Ministry of Science and Innovation.

## References

1. Houk, B., Martin, R.; Hochhaus, G.; Hughes, J. 2001, *Pharm. Res.*, 18, 67.
2. Dias, R. S.; Lindman B. (Eds). 2008, DNA Interactions with Polymers and Surfactants. Wiley Interscience, New Jersey.
3. Costa, D.; Morán, M. C.; Miguel, M. G.; Lindman, B. 2008, Cross-linked DNA Gels and Gel Particles, in DNA Interactions with Polymers and Surfactants, Dias, R. S.; Lindman, B (Eds), Wiley Interscience, New Jersey.
4. Lindman, B.; Dias, R. S.; Miguel, M. G., Morán, M. C., Costa, D. 2009, Manipulation of DNA by Surfactants, in Highlights in Colloid Science, Platikanov, D.; Exerowa, D (Eds), Wiley-VCH, Weinheim.
5. Morán, M. C.; Miguel M. G.; Lindman, B. 2007, *Langmuir*, 23, 6478.
6. Morán, M. C., Miguel, M. G.; Lindman, B. 2007, *Biomacromolecules*, 8, 3886.

7. Morán, M. C., Alonso, T.; Lima, F. S.; Vinardell, M. P.; Miguel, M. G.; Lindman, B. 2012, *Soft Matter*, 8, 3200.
8. Morán, M. C.; Infante, M. R.; Miguel, M. G.; Lindman, B.; Pons, R. 2010, *Langmuir*, 26, 10606.
9. Morán, M. C.; Miguel, M. G.; Lindman, B. 2011, *Soft Matter*, 7, 2001.
10. Morán, M. C.; Ramalho, A.; Pais, A.A.C.C.; Miguel, M. G.; Lindman, B. 2009, *Langmuir*, 25, 10263.
11. Morán, M. C.; Laranjeira, T.; Ribeiro, A.; Miguel, M. G.; Lindman, B. 2009, *J. Dispersion Sci. Technol.*, 30, 1494.
12. Morán, M. C.; Miguel, M. G.; Lindman, B. 2010, *Soft Matter*, 6, 3143.
13. Lapitsky, Y.; Kaler, E. W. 2004, *Colloids Surf. A.*, 250, 179.
14. Rigler, R.; Killander, D.; Bolund, L.; Ringertz, N. R. 1969, *Exp. Cell Res.*, 55, 215.
15. Ichimura, S.; Zama, M.; Fujita, H. 1971, *Biochim Biophys Acta.*, 240, 485.
16. Peacocke, A. R., 1973, The interaction of acridines with nucleic acids, in *Acridines*, Acheson, R. M. (Eds), Interscience Publishers, New York.
17. Darzynkiewicz, Z.; Traganos, F.; Sharpless, I.; Melamed, M. R. 1975, *Exp. Cell Res.*, 90, 411.
18. Cser, A.; Nagy, K.; Biczók, L. 2002, *Chem. Phys. Lett.*, 360, 473.
19. Crespi, C. L. 1995, *Adv. Drug Res.*, 26, 179.
20. Jondeau, A.; Dahbi, L.; Bani-Estivals, M. H.; Chagnon, M.C., 2006, *Toxicology*, 226, 218.
21. Martinez, V.; Corsini, E.; Mitjans, M.; Pinazo, A.; Vinardell, M. P., 2006, *Toxicol. Lett.*, 164, 259.
22. Fröhlich, E., Meindi, C.; Roblegg, E.; Griesbacher, A.; Pieber, T.R. 2012, *Nanotoxicology*, 6, 424.
23. Worle-Knirsch, J.M.; Pulskamp, K.; Krug, H.F. 2006, *Nano Lett.*, 6, 1261.
24. Monteiro-Riviere, N.A.; Inman, A.O.; Zhang, L.W. 2009, *Toxicol. Appl. Pharmacol.*, 234, 222.
25. Zhang, L.W.; Zeng, L.; Barron, A.R.; Monteiro-Riviere, N.A. 2007, *Int. J. Toxicol.*, 26, 103.
26. Stone, V.; Johnston, H.; Schins, R.P. 2009, *Crit. Rev. Toxicol.* 39, 613.
27. Hurt, R.H.; Monthieux, M.; Kane, A. 2006, *Carbon* 44, 1028.
28. Hasnat, M.A.; Uddin, M.M.; Samed, A.J.; Alam, S.S.; Hossain, S. 2007, *J. Hazard Mater.* 147, 471.
29. Xia, T.; Kovoichich, M.; Liong, M.; Zink, J.I.; Nel, A.E. 2008, *ACS Nano*. 2, 85.
30. Napierska, D.; Thomassen, L.C.; Rabolli, V.; Lison, D.; Gonzalez, L.; Kirsch-Volders, M.; Martens, J.A.; Hoet, P.H. 2009, *Small*, 5, 846.
31. Nogueira, D.R.; Moran, M.C.; Mitjans, M.; Martínez, V.; Pérez, L.; Vinardell, M.P. 2013, *Eur. J. Pharm. Biopharm.* 83, 33.
32. Warheit, D.B. 2008, *Toxicol. Sci.* 101, 183.
33. Teeguarden, J.G.; Hinderliter, P.M.; Orr, G.; Thrall, B.D.; Pounds, J.G. 2007, *Toxicol. Sci.* 95, 300.
34. Jones, C.F.; Grainger, D.W. 2009, *Adv. Drug Deliv. Rev.* 61, 438.
36. Pérez, L.; Pinazo, A.; García, M.T.; Lozano, M.; Manresa, A.; Angelet, M.; Vinardell, M.P.; Mitjans, M.; Pons, R.; Infante, M.R. 2009, *Eur. J. Med. Chem.*, 44, 1884.

37. Vinardell, M.P.; Benavides, T.; Mitjans, M.; Infante, M.R.; Clapés, P.; Clothier, R. 2008, *Food Chem. Toxicol.*, 46, 3837.
38. Sheetz, M. P.; Singer, S. J. 1974, *Proc. Natl. Acad. Sci. USA*1, 71, 4457.
39. Isomaa, B.; Hägerstrand, H., Paatero, G. 1987, *Biochim. Biophys. Acta*, 899, 93.
40. Vinardell, M. P.; Infante, M. R. 1999, *Comp. Biochem. Physiol. Part C*, 124, 117.
41. Morán, M. C.; Baptista, F. R.; Ramalho, A.; Miguel, M. G.; Lindman, B. 2009, *Soft Matter*, 5, 2538.