# 8 Cationic Lipospheres as Delivery Systems for Nucleic Acid Molecules

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## 8.1 INTRODUCTION

Gene delivery, or the release of exogenous genetic material into cells or tissues at a pathological state, has recently received much attention as a therapeutic methodology for a number of acquired and inherited diseases, including cancer [1–6]. Several diseases can, in fact, be traced back to defective or missing genes. Thus, bringing an appropriate gene into the appropriate cells could prevent or mitigate manifestations of the disease. *In vivo* cell transfection with foreign genes could be a promising pharmacologic treatment for a variety of diseases, including cancer, cystic fibrosis [7], and viral infections [6]. A large number of abnormally expressed genes have, in fact, been cloned and identified, allowing the prevention or the treatment of many human diseases [8], thus making gene therapy an active field that has progressed rapidly into clinical trials.

Because polydeoxyribonucleotides do not penetrate easily into the cell by themselves, the use of gene delivery vehicles has been proposed to facilitate the transport of genes to cellular targets. Gene transfer vehicles can be divided into viral and nonviral systems [9,10]. In the first case, the virus uses normal tactics to enter the cell and to begin to transcript its genome, but the absence of a key gene prevents

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the packaging of more infectious viral particles. At the same time, the gene of interest gets expressed by the cell and compensates for the lack of cellular proteins related to genetic defects. Viral delivery systems, such as retroviruses, adenoviruses, lentiviruses, and so forth, provide high transfection efficiency, and despite the deletion of vital genes, viruses can induce immune responses that are able to abolish transgenic expression. Moreover, viral vectors, because of their viral tropism, can infect only certain cells expressing receptors for proper replication, thus limiting their activity to specific tissues or compartments. In addition, engineered viruses may induce an immune response that compromises transfection resulting from subsequent injections and that lacks target specificity.

Obviating these problems, a large array of nonviral transfection agents have emerged as potential safe and effective gene vectors for *in vitro* applications. These nonviral systems include cationic lipids, peptides, glycopeptides, liposomes, micelles, glycosylated polymers, dendrimers, and micro- and nanoparticles [10–18]. The efficiency of nonviral transfection systems has, in general, improved several orders of magnitude in the last decade, even if none of them has yet proven to be efficient enough *in vivo* [10–18].

Thus, the key to success for any gene therapy strategy is to design a vector able to provide safe and efficient gene transcription of the transgene in a variety of cells and tissues. In this view, the development of protocols aimed at obtaining optimal and efficient genetic transfer has been studied [19–21] and has led to the production of many delivery vehicles that are able to bind to DNA. The optimal carrier has to accumulate at sites of diseases such as infections, inflammations, and tumors and has to be a small, neutral, and highly serum-stable particle. Moreover, it has to be not readily recognized by the fixed and free macrophages of the reticuloendothelial system.

Among nonviral systems, particulate carriers (e.g., polymeric nano- and microparticles, fat emulsion, liposomes) possess specific advantages and disadvantages. For instance, the relatively slow degradation of polymeric particles might possibly cause systemic toxic effects by impairment of the reticuloendothelial system or by accumulation at the injection site; cytotoxic effects have been indeed observed *in vitro* after phagocytosis of particles by macrophages and human granulocytes [20]. In addition, organic solvent residues deriving from preparation procedures, such as the solvent evaporation technique often used for liposome and polyester microparticles, could result in severe acceptability and toxicity problems [22].

However, with respect to other delivery systems, microparticles could maintain their physicochemical characteristics unaltered for long periods, allowing long-term storage; they can be administered through different ways (orally, intramuscularly, or subcutaneously), depending on their composition; and they are suitable for industrial production [23,24].

All together, these findings have encouraged the development of neutral and cationic lipospheres (CLS) as nonviral DNA-mediated gene transfer techniques because CLS enable the extemporaneous production of pharmaceutical formulations. Like emulsions and liposomes, lipospheres (LS) consist of physiologically well-tolerated ingredients that have often already been approved for pharmaceutical use

in humans [25]; in addition, similar to polymeric nanoparticles, their solid matrix can protect drugs against chemical degradation and allow modulation of drug release profile [24]. Thus, LS combine the advantages of polymeric nanoparticles, fat emulsions, and liposomes, avoiding some of their typical disadvantages, such as cytotoxic effects after phagocytosis, toxic effects of organic residues after the production of polymers, and lack of large industrial-scale production.

LS and CLS are solid microparticles with a mean diameter usually between 0.2 and 500 μm, composed of a solid hydrophobic fat matrix in which (in the case of LS) the bioactive compound or compounds are dissolved or dispersed. Because of their large range in particle size, LS can be administered by different routes, such as orally, subcutaneously, intramuscularly, or topically, or they can be used for cell encapsulation, thus allowing them to be proposed for treatment of a number of diseases [26–28]. The *in vivo* distribution of LS demonstrated a high affinity to vascular wells (including capillaries), to inflamed tissues, and to granulocytes [29,30].

In addition, LS have several advantages over other delivery systems: good physical stability, low cost of ingredients, ease of preparation and scale-up, and high entrapment yields for hydrophobic drugs. Moreover, LS have been successfully used both for the controlled delivery of various types of drugs and as carriers of vaccines and adjuvants [29,31,32].

This chapter will discuss the production and characterization of CLS obtained by different techniques, the formation of the complex between CLS and DNA, and, finally, the *in vitro* cytotoxicity on different cell lines and the transfection efficiency of CLS.

## 8.2 NUCLEIC ACID STABILITY: GENERAL CONSIDERATIONS AND IMPROVEMENT

In the past few years, many types of nucleic acid molecules, such as synthetic oligonucleotides complementary to viral or eukaryotic RNA, have been reported to inhibit viral replication in cell culture as well as the *in vitro* and *in vivo* expression of genes [33,34], thus indicating the use of synthetic antisense DNA for the modulation of specific gene expression as a novel pharmacological approach for pathological states deriving from an altered expression of a gene or genes. In this respect, viral infections, including AIDS as well as neoplastic diseases, could represent possible targets for oligonucleotide therapeutics [35]. Moreover, other DNA molecules — such as triple helix–forming oligonucleotides and double-stranded polymerase chain reaction–generated DNA fragments, mimicking genomic regulatory regions recognized by transcriptional factors — could be efficiently employed molecular tools to study and modulate gene transcriptional activity [36].

Nevertheless, in spite of these interesting pharmacobiological properties, nucleic acid molecules (i.e., oligonucleotides and polymerase chain reaction–generated DNA fragments) are, in general, rapidly degraded by cellular and extracellular nucleases. It is thus necessary that, to carry on their pharmacobiological activities, nucleic acid molecules should remain stable after *in vivo* administration, retaining an appreciable

half-life in the extracellular environment. The stability problems of this class of compounds have been approached largely through chemical modification of the oligonucleotides, and mainly through substitution of the natural phosphodiester linkage with alternative chemical groups [37]. Although many backbone-modified oligonucleotides have been synthesized and proposed, methylphosphonates and phosphorothioates are the most studied and used nuclease-resistant compounds [37,38].

An alternative approach to chemical modification of oligonucleotides is offered by attaching a "pendant" group to the 5- or 3-ends of the oligonucleotide [33]. A large variety of pendant groups has been designed and synthesized, both to study oligonucleotide cellular uptake and compartmentalization, such as fluorochrome, and to improve or modify the biological activity of the oligonucleotides. This latest class comprises intercalating agents playing different roles, such as stabilizing intercalating agents, cleaving or photo-induced cleaving reagents, and photo-induced cross linkers [39], as well as lipophilic groups such as fatty acid or cholesterol moieties [40].

The potential of the chemically modified nucleic acid molecules has been proven by *in vitro* studies; however, the *in vivo* therapeutic applicability of these molecules seems to be unsatisfactory because of their possible toxic effects (largely unknown) and adverse bioavailability. In this view, both antisense and transfection technologies require reliable and efficient systems for their delivery into target cells. On the basis of this consideration, the development of an efficient nucleic acid delivery system represents one of the key steps for these therapeutic agents, which are necessary for a practical clinical utilization of natural or unnatural oligonucleotides.

In this respect, an interesting approach to reduce degradation and possible toxicity problems related to nucleic acid use *in vivo* is offered by their encapsulation in or association to microcarrier systems, such as neutral or cationic liposome and polymeric microparticles [41–44].

Microparticles can, in principle, vehiculate nucleic acids in two ways: DNA can be physically entrapped in the polymeric matrix of the particle, or DNA can be bound through electrostatic interactions to the positively charged surface of cationic particles. The surface of these carriers could be positively charged as a result of the presence of quaternary ammonium cationic lipids in the liposome composition, such as DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) [43,45], DEBDA (diisobutyl-cresoxy-ethoxy-ethyl-dimethyl-benzyl-ammonium hydroxide) [46], or CTAB (cetyltrimethylammonium bromide) [47]. In this way, negatively charged nucleic acids are able to complex the surface of preformed cationic carrier. The association of DNA molecules to preformed cationic microparticles could provide two important benefits. First, DNA is not exposed to the chemical, thermal, or mechanical stresses often present in the production of microparticles, and second, the association of DNA to microparticles can be performed extemporarily immediately before their administration [45,48]. In this way, both microspheres and DNA can be maintained separately in sterile lyophilized forms, thus avoiding possible long-term instability problems. To confer a positive charge to the microparticle surface, different approaches can be proposed, such as a polymeric mixture

that is constituted of an uncharged copolymer and a cationic one, or a not entirely polymeric mixture constituted of a neutral copolymer plus a cationic lipid [49,50].

As above reported, among microparticles, CLS have been proposed as a new type of fat-based encapsulation system developed for drug delivery of bioactive compounds.

## 8.3 PREPARATION OF CLS

### 8.3.1 Materials and Preparation Methods

CLS can potentially be used for gene delivery and have shown to be less rapidly cleared from the circulation than negatively charged particles [51]. In addition, the choice of the lipid matrix also plays an important role on the morphology of the particles and on the possible formation of aggregates that are important for the circulations of this carrier [52]. As described in many papers [53–58], LS, and thus also CLS, can be prepared in a variety of ways by using a wide range of chemical ingredients [26]. To obtain a formulation suitable for human administration, triglycerides and monoglycerides have been chosen as LS biomaterials because of their high biocompatibility, high chemicophysical stability, and drug delivery release. However, all preparation methods make use of surfactants, and the resulting particles are characterized by an overall positive, neutral, or negative surface charge, which is determined by the composition, influencing the aggregation tendency in suspension. The simplest and more used preparation methods are typified by the emulsion–melt dispersion and the solvent evaporation technique [53].

It has to be underlined that, in comparison to LS containing nucleic acid molecules inside the particle, the production of CLS may be performed obviously without considering the stability problems of nucleic acid molecules. In this view, some preparation procedures are not considered, such as the microemulsion technique [55] that represents a favorable method when working with substances unstable because of the high mechanical stress produced by high-pressure homogenization.

The production of CLS by the melt dispersion technique is based on the melting of the lipid core material together with the lipophilic agent (i.e., phospholipids). Afterward, a warm aqueous solution is added to the molten material and is mixed by various methods (i.e., mechanical stirring, shaking, sonication, homogenization). Then the preparation is rapidly cooled until lipid solidification and the formation of particle dispersion. This method was used by Olbrich et al. [19] to produce the cationic solid lipid nanoparticles to use as novel transfection agent.

The solvent evaporation technique is based on the use of organic solvents as dissolving agents for the lipid matrix (i.e., phospholipids or triglycerides and monoglycerides) and the subsequent evaporation of the solvent within an aqueous medium until a CLS dispersion is obtained.

However, Erni et al. [50] have prepared LS with a method established in their laboratory using a solvent extraction. In particular, the method is based on the dissolution of the triglyceride (i.e., tripalmitin) and the cationic lipid in the organic solvent (i.e., dichloromethane), and on the addition of an aqueous polyvinyl alcohol (PVA)

solution (0.5% w/w) used as extraction fluid. The solution and the extraction fluid are pumped into a static microchannel mixer, leading to the production of an O/W (oil in water) emulsion. The mixing leads to the production of fine lamellae, which subsequently disintegrate into droplets, allowing the formation of solid lipid microparticles dispersed in the extraction aqueous medium.

In a recent set of experiments [56], LS composed of triglycerides and monoglycerides were alternatively produced by melt dispersion technique, by solvent evaporation, or by the w/o/w double-emulsion method. The influence of preparation parameters, such as type and amount of lipids, presence and concentration of surfactants, stirring speed, and type of stirrer was studied. In the case of LS prepared by melt dispersion, the use of a lipid composition of cetyl alcohol/cholesterol (2:1, w/w), a 5% (w/w) gelatin solution (50 Bloom grades), and a 1000-rpm stirring speed resulted in the production of spherical particles with a high percentage of recovery (82%, w/w), a mean diameter of 80 μm, and a narrow size distribution. In the case of LS prepared by solvent evaporation, the best results in terms of LS morphology, recovery, and size distribution were obtained by the use of a lipid composition of tristearin/monostearate (66:34, w/w), a 1% (w/w) PVA solution, a 750-rpm stirring speed, and a 55-mm three-blade turbine rotor.

The solvent evaporation method resulted in the production of LS characterized by a smaller size (20 μm mean diameter) but poor mechanical properties in respect to particles with the same composition that were obtained by the melt dispersion technique (170 μm mean diameter). The use of a combination of lipids and a methacrylic polymer (Eudragit RS100) overcame this problem, resulting in the production of spherical particles with a narrower size distribution and good mechanical properties [53,56].

Concerning the SLN produced by hot homogenization as described by Olbrich et al. [19], as lipidic matrix Compritol ATO 888 or paraffin were used, as tenside a mixture of Tween 80 and Span 85 was used, and as charge carrier either EQ1 [*N*,*N*-di-(β–steaorylethyl)-*N*,*N*-dimethylammonium chloride] or cetylpyridinium chloride were used. The resulting particles were characterized by size between 101 and 105 nm and showed zeta potentials around 40 mV at pH 7.4.

In contrast, solid lipid microparticles consisting of a tripalmitin matrix and cationic lipids prepared using the micromixer-based solvent extraction process as described by Erni et al. [50] were of monomodal size, showing a narrow size distribution in the submicrometer range (Table 8.1).

### 8.3.2 Formation of CLS/DNA Complex

To our knowledge, only a few papers have been recently published concerning CLS for gene therapy. In particular, to investigate the ability of CLS to bind nucleic acids, different nucleic acid molecules were considered as models, namely, two double-stranded plasmid DNA [19,50] and defibrotide (DFT, Mw 26,200 Da), a single-stranded polydeoxyribonucleotide (DNA) sodium salt extracted from mammalian organs [56]. This last molecule was chosen because it is included in a pharmaceutical product presently on the market as an antithrombotic agent, it is a low–molecular weight nucleic acid mimicking the behavior of polymerase chain reaction products

**TABLE 8.1**
**Composition, Particle Size, Zeta Potential, and Loading Efficiency of Nucleic Acid of the Cationic Lipospheres Considered in the Chapter**

| Cationic Lipospheres Composition (% of component) | Method of Preparation | Particle Size (nm) | Zeta Potential (mV) | Reference |
|---|---|---|---|---|
| Solid paraffin (4%), Tween 80/Span 65 (7:3) (4%), cetyltrimethylammonium bromide (0.5%) | Melt dispersion | 103 ± 2.3 | +41.2 ± 0.9 | [19] |
| Compritol (4%), Tween 80/Span 65 (7:3) (4%), cetyltrimethylammonium bromide (0.5%) | Melt dispersion | 105 ± 1.7 | +40.2 ± 0.5 | [19] |
| Compritol (4%), Tween 80/Span 65 (7:3) (4%), N,N-di-(β-steaorylethyl)-N,N-dimethylammonium chloride (1%) | Melt dispersion | 101 ± 2.3 | +42.3 ± 1.0 | [19] |
| Stearylamine (1.8%), cetyltrimethyl-ammonium bromide (1.2%), Trimyristin (1.8%), Polysorbate 80 (19.0%) | Melt dispersion (unwashed) | 189.5 (bimodal) | +17.9 | [54] |
| Stearylamine (1.8%), cetyltrimethyl-ammonium bromide (1.2%), Trimyristin (1.8%), Polysorbate 80 (19.0%) | Melt dispersion (dialyzed) | 211.7 | 10.1 | [54] |
| Tripalmitin (95%), cetyltrimethyl-ammonium bromide (5%), PVA (0.5%) | Solvent extraction | 1890 | +21.4 ± 7.3 | [50] |
| Tripalmitin (95%), $DDAB_{12}$ (5%) PVA (0.5%) | Solvent extraction | 5150 | +24.9 ± 7.9 | [50] |
| Tripalmitin (95%) DDAB18 (5%) PVA (0.5%) | Solvent extraction | 2370 | +30.9 ± 8.7 | [50] |
| Tripalmitin (95%) PVA (0.5%) | Solvent extraction | 6830 | 8.4 ± 2.3 | [50] |
| Tristearin (66%) Glyceryl monostearate (33%) cetyltrimethyl-ammonium bromide (5%) PVA (1%) | Melt dispersion | 1260 ± 150 | n.d.[a] | [56] |
| Tristearin (66%) Glyceryl monostearate (33%) $DDAB_{12}$ (5%) PVA (1%) | Melt dispersion | 1650 ± 90 | n.d. [a] | [56] |
| Tristearin (66%), Glyceryl monostearate (33%), $DDAB_{18}$ (5%), PVA (1%) | Melt dispersion | 1420 ± 210 | n.d. [a] | [56] |

[a] n.d.: not determined

and synthetic oligonucleotides, and its complex with cationic liposomes is a patented pharmaceutical formulation.

The formation of the CLS/nucleic acid complex was performed by mixing an aqueous suspension of CLS with a solution containing the nucleic acids [19,50,56].

In particular, Erni et al. [50] used a gentle shaking in phosphate-buffered salt with pH 7.4 or Dulbecco's Modificatin of Eagle's Medium (DMEM) for 2 h at 41°C, whereas Olbrich et al. [19] prepared the CLS/DNA complexes by mixing 20 mg/mL of plasmid in 200 mL of 25 mM Hepes (pH 7.4).

In the first case [50], the loading efficiency was in the range of 65 to 95% for CLS and was up to a maximum of 56% for neutral particles, showing that adsorption was enhanced by the cationic surface of the particles. In addition, it was proven that loading efficiency was further dependent on the composition of the medium used for dispersing the particles. With respect to the second case [19], the binding of the CLS to polyanionic DNA was studied by analysis of the electrophoretic mobility of the DNA within an agarose gel, the so-called electrophoretic mobility shift assay. Addition of the tenside mixtures or the cationic modifier molecules alone did not result in a change of DNA migration during electrophoresis of the cationically modified CLS; however, one mixture (SII-13) resulted in a shift in DNA mobility, whereas the other CLS formulations (SII-4, SII-5, SII-9, SII-10), as well as the surfactant mixture (SII-17), were not able to immobilize detectable amounts of DNA at w/w ratios up to 10,000. At ratios above 50, 100% of the DNA was shifted to a higher apparent molecular weight or was even completely immobilized within the wells. Twenty to 50 weight equivalents were sufficient to bind most of the DNA, and five or fewer weight equivalents of SII-13 were practically inactive.

Concerning DFT association to CLS (third case) [56], Figure 8.1 shows the ability of the different types of CLS to ionically bind the nucleic acid when used at a different positive to negative molar charge ratio (+/–) comprised between 1:1 to 16:1. In particular, CLS containing $DDAB_{18}$ (dioctadecyl-dimethylammonium bromide) prepared at 500 rpm in the presence of PVA (white diamond) or gelatin 50 Bloom (black diamond), both at 1% w/v, were considered. As reported in Figure 8.1A, the association of DFT to CLS of both types showed a similar trend, reaching at the highest molar charge ratio an association around 90%, namely, 87.3% and 90.4% for CLS obtained with PVA and gelatin 50 Bloom, respectively. The association ability of CLS containing $DDAB_{12}$ (didodecyl-dimethylammonium bromide) and CTAB were evaluated only on lipoparticles obtained in the presence of PVA. As demonstrated by the data of Figure 8.1B for $DDAB_{12}$ and CTAB, the association capacity of CLS was only scarcely affected by their size; in fact, smaller particles (mean size 0.66 μm) displayed an association profile almost identical to that of larger particles (mean size 0.87 μm).

Cortesi et al. [56] have conducted a study to evaluate the strength of the interaction occurring between DFT and CLS and to evaluate whether different cationic detergents could cause a variation in binding strength. Briefly, CLS containing increased concentrations of cationic lipid were incubated with DFT for 10 min, and then samples were electrophoresed to determine the electrophoretic migration of DNA complexed to liposome. The results reported in Figure 8.2 indicate that DFT migration is only slightly retarded by low amounts of CLS, whereas higher CLS concentration (especially in the case of $DDAB_{18}$) causes the formation of high–molecular weight complexes with DFT molecules that precipitated within the well. These nonmigrating complexes were attributed to interparticle bridges formed by DNA molecules [59].

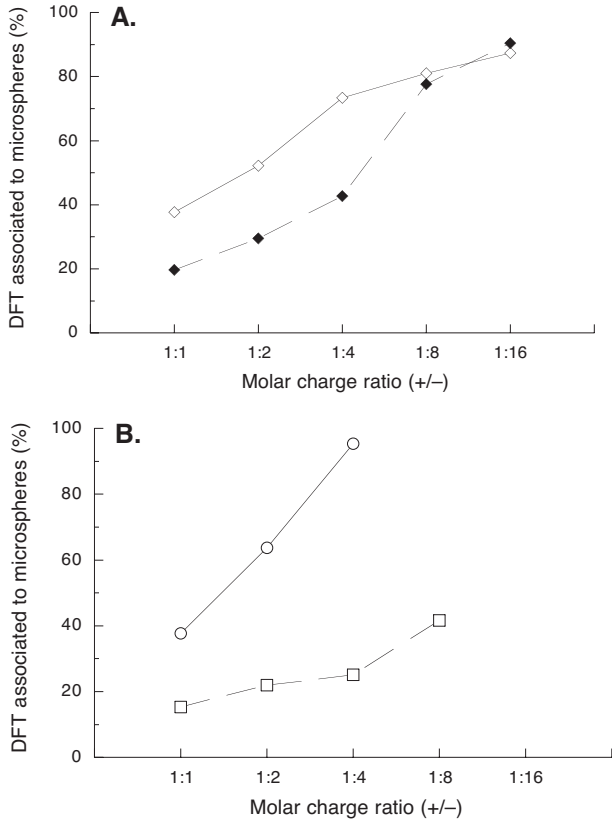


**FIGURE 8.1** Percentages of DFT association to CLS containing as cationic lipid. **(A)** $DDAB_{18}$ prepared in the presence of PVA (open diamonds) or gelatin (closed diamonds); **(B)** $DDAB_{12}$ (open circles) or CTAB (open squares).

## 8.4 CYTOTOXICITY OF NUCLEIC ACIDS FROM CLS

To evaluate the cytotoxic activity of CLS, different types of experiments were performed. For instance, Erni et al. [50] tested the cytotoxicity of the different cationic lipids used for the preparation of CLS in two different cell lines (293 embryonic kidney cells and RAW macrophages) for compromised membranes of dead cells indicating necrosis [60]. All cationic lipids displayed comparable concentration-dependent cytotoxicity profiles, all being nontoxic at concentrations up to 2 mg/well. Slightly increased cytotoxicities were observed with the short alkyl chain–length $DDAB_{12}$. Interestingly, a lower necrosis was observed in the RAW macrophage cell line as compared to the 293 cell line. Significant cytoxicity was found in primary macrophages after adding CLS or cationic lipid at amounts that did not cause detectable cytotoxicity in 293 cells or RAW macrophages. In addition, the cytotoxicity of the various CLS formulations turned out to be comparable to the

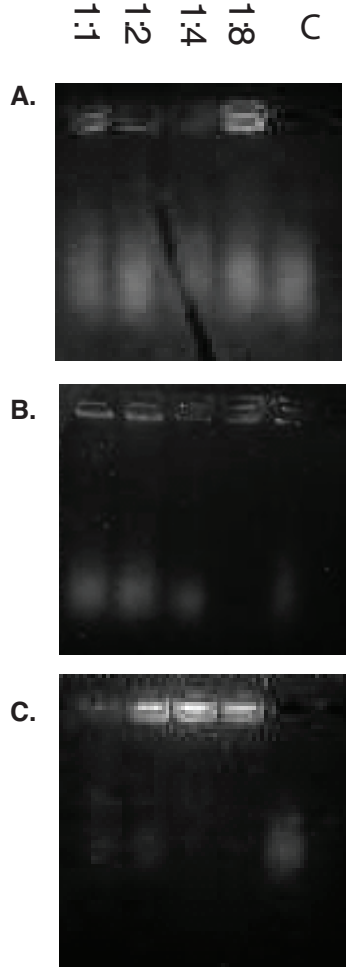


**FIGURE 8.2** Effect of CLS complexation on the electrophoretic migration of DFT. **(A)** $DDAB_{18}$, **(B)** $DDAB_{12}$, **(C)** CTAB. The following CLS/DFT charge molar ratios, namely, 1:1, 1:2, 1:4, and 1:8 mol/mol, were used. C: control untreated DFT.

respective soluble cationic lipid, with negligible effects at concentrations less than 2 mg/well (referring to the amount of cationic lipid incorporated in the formulation). Neutral lipid particles prepared under identical conditions without the addition of cationic lipid did not show any detectable cytotoxicity when added to the cells in comparable amounts.

Olbrich et al. [19] tested *in vitro* the cytotoxicity of the transfection agents, considering the viability of Cos-1 cell monolayers. Both the initial perturbation of cell integrity during the 4-h incubation and the influence on the cellular activity after 48 hours were assessed by measuring the release of lactate dehydrogenase and the mitochondrial conversion of the tetrazolium salt WST-1. Unmodified paraffin particles (SII-4) did not show cytotoxicity in the LDH (lactate dehydrogenase) release

assay. The same behavior was observed for paraffin particles modified with EQ1 (SII-5), whereas those containing cetylpyridinium resulted in 50% LDH release at 120 µg/mL. Similar observations were made for modified Compritol-based particles with no detectable LDH release after incubation with up to 3 mg/mL SII-13 (EQ1 modified) but a $LD_{50}$ of 150 µg/mL for SII-10, which contains the modifier cetylpyridinium chloride. Interestingly, the mixture of Tween/Span and EQ1 (SII-17) was more toxic when it was not bound to a suitable particulate matrix, as in batch SII-13. Assessment of the cell viability after 48 h, using the WST-1 test, was generally more sensitive than the LDH release assay. However, it produced the same overall correlations: cetylpyridinium-containing SLN were toxic, and EQ1-modified particles were less problematic. Moreover, modification of paraffin-based SLN with EQ1 reduced their negative effect on cell viability, resulting in $IC_{50}$ values of 700 mg/mL instead of 100 mg/mL. The pure tenside mixture (SII-17) showed similar $IC_{50}$ values of 150 mg/mL.

To determine the cytotoxic activity of cationic microparticles, Cortesi et al. [56] performed an *in vitro* study treating human leukemic K562 and murine macrophagic J774 cell lines with different amounts of CLS. After 6 d in cell culture, cells were electronically counted. Figure 8.3 reports the cytotoxic activity of CLS containing, alternatively, $DDAB_{18}$, $DDAB_{12}$, and CTAB (with an amount between 0 and 300 µg/mL corresponding to a cationic lipid concentration between 0 and 250 µM). The obtained data demonstrated that cationic lipoparticles are only slightly cytotoxic, especially when compared with other cationic formulations used for gene therapy, such as liposomes [49], indicating that $DDAB_{18}$-based lipoparticles could be safely used in *ex vivo* experiments. To assess whether cationic lipoparticles can be efficiently internalized by *in vitro* cultured cells, an experiment was also conducted with J774 murine macrophages. Cells were cultured in the presence of CLS and, after 5 min, were fixed with glutaraldehyde and observed by scanning electron microscopy analysis. As clearly evident in Figure 8.4, CLS are efficiently internalized (probably by phagocytosis) in J774 cells, indicating that they could be used as a delivery system for at least *ex vivo* experiments.

Erni et al. [50] have demonstrated that neutral lipid particles consisting of a tripalmitin matrix can be efficiently phagocytosed by primary macrophages *in vitro*. In particular, complete intracellular degradation was observed within 24 h, making neutral lipid particles a suitable carrier for the immediate delivery of therapeutics to antigen-presenting cells. However, CLS also adsorbed plasmid DNA and triggered the cellular internalization of the macromolecules by phagocytic macrophages. Surprisingly, the CLS also triggered the internalization of these molecules by nonphagocytic 293 cells. This was probably a result of the detachment of nanocomplexes formed of cationic lipid and DNA from the surface of DNA-loaded CLS and their subsequent uptake into the cells.

## 8.5 TRANSFECTION EFFICIENCY

Erni et al. and Olbrich et al. [19,50] have data about transfection performed with CLS as carriers. For instance, Erni et al. tested the transfection efficiency in primary macrophages and in RAW macrophages. No transfection was observed during 72 h

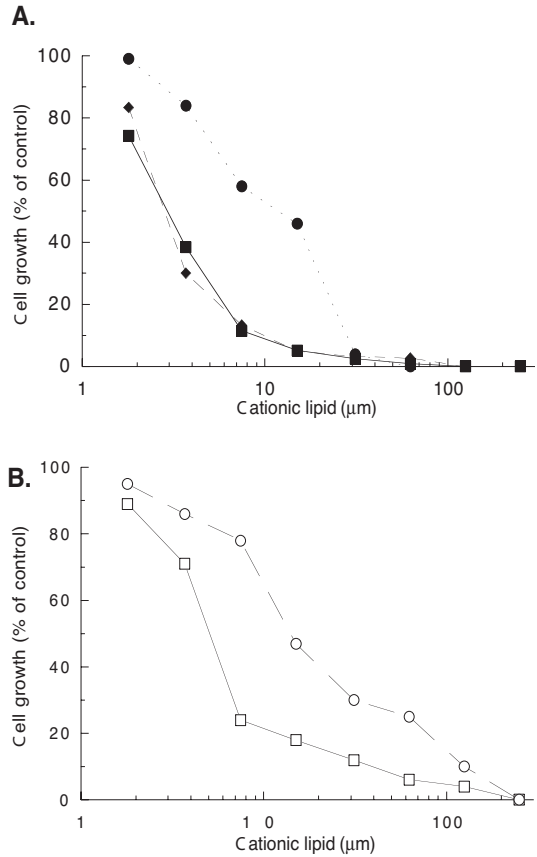


**FIGURE 8.3** Cytotoxic activity of $DDAB_{18}$ (circle), $DDAB_{12}$ (square), and cetyltrimethylammonium bromide (diamond) cationic lipoparticles on the cultured human K562 **(A)** and murine macrophagic J774 **(B)** cell lines. Data represent the percentage of cell number per milliliter compared with untreated control K562 cells.

in either type of cells. However, the authors demonstrated that transfection efficiency of the DNA-loaded CLS was most pronounced in nonphagocytic cells and was not detected in the macrophage cell line or in primary macrophages. Further studies have revealed that cytotoxic effects of CLS were more pronounced in the phagocytic cells, because of the very rapid uptake and degradation of the CLS in these cells. In particular, free cationic lipid equivalent to the amount of cationic lipid contained in the tested CLS was mixed with DNA and administered to the various cell cultures as controls. Detectable transfection in 293 cells was exclusively found with $DDAC_{18}$. Its level was about threefold higher than with CLS containing $DDAC_{18}$ and was in the range of the commonly used transfectant DOTAP. CTAB and $DDAC_{12}$ failed to induce cell transfection. Thus, transfection mediated by CTAB or $DDAC_{12}$ was only feasible when enhanced with CLS. However, when CLS containing $DDAC_{18}$ were separated by filtration, and the supernatant subsequently mixed with DNA and further

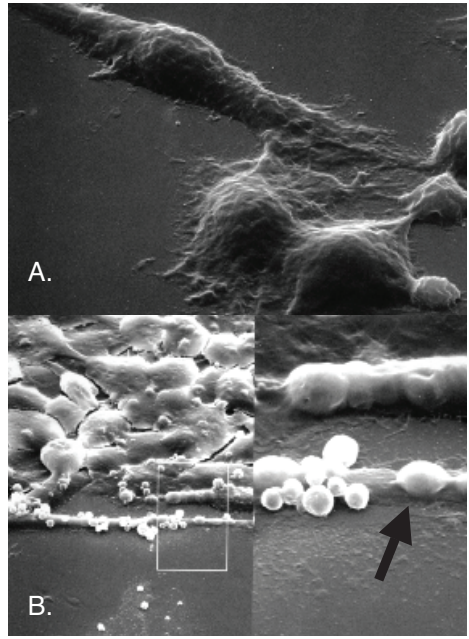


**FIGURE 8.4** Scanning electron micrograph of J774 cells alternatively untreated **(A)** and treated **(B)** with cationic lipospheres containing $DDAB_{18}$. Black arrows indicate internalized submicron particles. Bar corresponds to 10 and 30 μm in panels A and B, respectively.

used for transfection, no measurable transfection occurred. This may indicate the absence of significant amounts of free $DDAC_{18}$ in the dispersion medium that are available for complex formation with DNA that induces cell transfection.

As previously stated, Olbrich et al. [19] tested both cytotoxicity and transfection efficiency on Cos-1 cells *in vitro*. In particular, the ability of CLS to transfect the pCMVb reporter gene plasmid at a fixed concentration into Cos-1 cells was tested in the absence or in the presence of 100 mM of the endosomolytic agent chloroquine. The obtained results demonstrated that CLS were able to promote transfection in a wide window of CLS/DNA ratios, whereas incubation with uncomplexed DNA did not result in detectable levels of β-galactosidase expression in this experimental setup. In the absence of chloroquine, the strongest reporter gene expression was observed when the DNA was complexed with 60 weight equivalents of CLS, although, when the overall transfection efficiency of CLS is compared to established transfection agents such as poly-L-lysine or polyethylenimine, it has to be ranked only moderate. Nevertheless, the controls, SII-10 (a CLS not able to immobilize plasmid DNA in the agarose gel) and SII-17 (the pure cationic modifier unable to stably bind DNA), were not able to retransfect Cos-1 cells. In all cases, the β-galactosidase activity was within the background range.

Another important aspect of transfection agents, especially for nonviral systems, was the efficiency/toxicity ratio. The CLS described by Olbrich et al. [19] showed only moderate transfection efficiencies compared with the established polymers

poly-L-lysine and polyethylenimine. However, this transfection activity was accompanied only by a low degree of cytotoxicity. A number of possibilities exist for optimizing the activity of the system, including choice of matrix lipid, size, and modifier.

## 8.6 CONCLUSIONS

During recent years, solid lipid nanoparticles have attracted increasing attention. However, only a few studies that have been aimed to obtain innovative nonviral transfection systems for gene therapy have been performed on CLS. In the last decade, the efficiency of nonviral transfection systems has improved several orders of magnitude. Although as yet none has proven to be effective enough *in vivo*, new developments are still ongoing. Among nonviral transfection systems, colloidal carriers such as CLS represent an alternative drug delivery system to emulsions, liposomes, and polymeric particles. From the analysis of the results reported in this chapter, it emerges that CLS may provide a new, efficient means for the immediate intracellular delivery of therapeutic macromolecules. Nevertheless, caution is warranted for cationic carriers, which may accentuate cytotoxic effects in the phagocytic cells.

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