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Graphical abstract

Adsorption of DNA onto anionic lipid surfaces

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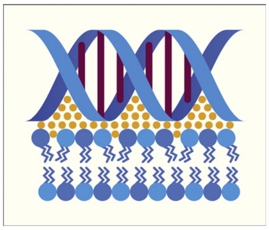

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Highlights

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Adsorption of DNA onto anionic lipid surfaces

Alberto Martín-Molina [a,*], Germán Luque-Caballero [a], Jordi Faraudo [b], Manuel Quesada-Pérez [c], Julia Maldonado-Valderrama [a]

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- Anionic lipoplexes are considered to be promising tools for gene therapy because of their low cytotoxicity.
- The DNA adsorption onto anionic lipid surfaces is mediated by multivalent cations.
- Langmuir lipid monolayers serve as models to study DNA adsorption onto lipid membranes.
- Computer simulation addresses specifically the molecular mechanism underlying the binding of DNA with anionic lipids.

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Historical perspective

Adsorption of DNA onto anionic lipid surfaces

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ABSTRACT

Currently self-assembled DNA delivery systems composed of DNA multivalent cations and anionic lipids are considered to be promising tools for gene therapy. These systems become an alternative to traditional cationic lipid–DNA complexes because of their low cytotoxicity lipids. However, currently these nonviral gene delivery methods exhibit low transfection efficiencies. This feature is in large part due to the poorly understood DNA complexation mechanisms at the molecular level. It is well-known that the adsorption of DNA onto likely charged lipid surfaces requires the presence of multivalent cations that act as bridges between DNA and anionic lipids. Unfortunately, the molecular mechanisms behind such adsorption phenomenon still remain unclear. Accordingly a historical background of experimental evidences related to adsorption and complexation of DNA onto anionic lipid surfaces mediated by different multivalent cations is firstly reviewed. Next, recent experiments aimed to characterise the interfacial adsorption of DNA onto a model anionic phospholipid monolayer mediated by Ca^{2+} (including AFM images) are discussed. Afterwards, modelling studies of DNA adsorption onto charged surfaces are summarised before presenting preliminary results obtained from both CG and all-atomic MD computer simulations. Our results allow us to establish the optimal conditions for cation-mediated adsorption of DNA onto negatively charged surfaces. Moreover, atomistic simulations provide an excellent framework to understand the interaction between DNA and anionic lipids in the presence of multivalent divalent cations. Accordingly, our simulation results in conjunction go beyond the macroscopic picture in which DNA is stuck to anionic membranes by using multivalent cations that form glue layers between them. Structural aspects of the DNA adsorption and molecular binding between the different charged groups from DNA and lipids in the presence of divalent cations are reported in the last part of the study. Although this research work is far from biomedical applications, we truly believe that scientific advances in this line will assist, at least in part, in the rational design and development of optimal carrier systems for genes and applicable to other drugs.

Contents

Abbreviations: AFM, Atomic force microscopy; BAM, Brewster angle microscopy; CCC, Critical coagulation concentration; CG, Coarse grain; CHO, Cholesterol; CHO-K1, Chinese hamster ovary-K1; −COO, Carboxylic acid; DCP, Dicetylphosphate; DSC, Differential scanning calorimetry; DMPE, 1,2-Dimyristoylphosphatidylethanolamine; DNA, Deoxyribonucleic acid; DOPC, Dioleoyl phosphatidylcholine; DOPE, 1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine; DOPG, Dioleoylphosphatidylglycerol; DOPS, Dioleoyl phosphatidylserine; DPPC, Dipalmitoyl phosphatidylcholine; DPPS, Dipalmitoyl phosphatidylserine; DPPG, Dipalmitoylphosphatidylglycerol; dsDNA, Double stranded DNA; ds:RNA, Double stranded RNA; FENE, Finite extensible nonlinear elastic; FTIR, Fourier-transform infrared spectroscopy; HM, Heating method; IRRAS, Infrared reflection absorption spectroscopy; LJ, Lennard Jones; LUV, Large unilamellar vesicles; M^{2+}, Metal divalent cation; MC, Monte Carlo; MD, Molecular dynamics; MG, Malachite green; MLV, Multilamellar vesicles; N-C12-DOPE, N-dodecanoyl dioleoyl phosphatidylethanolamine; NR, Neutron reflectometry; PB, Poisson–Boltzmann; PC, Phosphatidylcholine; PO_4, Phosphate; POPA, 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphate; POPN, 1-Palmitoyl-2-oleoylphosphatidyl-nucleosides; POPC, Palmitoyl-oleoyl-phosphatidylcholine; PS, Phosphatidylserine; QCM-D, Dissipative quartz crystal microbalance; RNA, Ribonucleic acid; SAXS, Small angle X-ray scattering; SHG, Second harmonic generation; siRNA, Short interfering RNA; ssDNA, Single stranded DNA; ssRNA, Single stranded RNA; tRNA, Transfer RNA; SPM, Scanning probe microscopy; VMD, Visual molecular dynamics; VSFG, Vibration sum frequency generation.

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1. Introduction and background

Gene therapy is considered one of the most strategic scientific methods to introduce genetic material in damaged cells (target cells) to repair or replace damages in the cellular genetic material and thus to relieve symptoms of many inherited or acquired human diseases. In contrast to traditional medicine, the purpose of gene therapy is to cure diseases at a molecular level, repairing possible damages in the cellular DNA and preventing their appearance. In particular, non-viral gene delivery vectors represent one of the strategies most successful in gene therapy at present, given that they are biocompatible systems with low cytotoxicity. Since the early 60s, the research aims to combine the DNA with chemical substances which are able to electrically neutralize, complexate and condensate, the DNA [1–3]. To this end, essays with polypeptides such as polyarginine or polihystidine have been carried out but their elevated toxicity prevents their use in transfection *in-vivo*. Enhanced results are provided by using polylysine with their monomers in L configuration, since this biodegraded polypeptide is less toxic. Other studies performed with polycations such as spermidine or spermine, do not show significant improvements in the transfection efficiency by direct injection. Also, cationic polymers such as polivinilamine and poli-vinilpiridine have been tested. These are positively charged polyelectrolytes in physiological media which can complexate the DNA that will be extended once in the cytoplasm. However, their transfection efficiency has not been yet proved yet. Finally, dendrimers, with structures similar to that found in histones, are efficient only *in-vitro*. Since the early 1980s, chemical transfection methods, based on liposome vectors, emerge as an alternative to overcome the lack of efficiency found in the rest of the chemical methods. In fact, lipoplexes (liposomes–DNA) are the most used non-viral vectors in transfection due to their capability to protect the gene and introduce it into the target cell. However, despite great progresses in relation to the use of lipoplexes in gene therapy, the use of these gene vehicles is still in an early stage principally due to their low transfection capacity with regard to the viral vectors. Nonetheless, as predicted by Danilo D. Lasic (1952–2000), pioneer of the application of liposomes in medicine, a good understanding of the physicochemical properties of the lipoplexes contributes significantly to the increase in their rates of transfection [1]. In fact, cationic lipids complexate the DNA giving rise to a great variety of supramolecular structures (cationic lipoplexes) where the cationic lipid bilayers appear as the polycations with the capability to compact the DNA and to form a supramolecular assembly with applications in gene delivery [4–9]. These complexes facilitate the transfection owing to the similarity between the cell membrane and the bilayer lipid membrane which yields endocytosis (process whereby cells absorb material from the outside). However, cationic liposomes induce a large number of adverse effects for DNA delivery. For instance, the use of cationic liposomes to deliver nucleic acids to cells is inappropriate due to the lack of efficiency in the transfection, the instability of storage, the potential toxicity of the cationic lipid in a cellular environment [10–17], *etc*. Cationic lipoplexes can be inactivated in the presence of serum and they are not likely to maintain their stability upon intravenous injection as a consequence of the presence of high concentrations of negatively charged plasma proteins and circulating blood cells [13,15]. Regarding the toxicity, experimental studies have shown that they cause cytotoxicity in both, *in-vitro* and *in-vivo* [10–12,14,16,17]. For instance, Filion and Philips studied cationic lipoplexes that include the zwitterionic lipid: DOPE as a helper lipid (frequently used to form cationic lipoplexes [18–21]). In particular, these authors evaluated, among other things, the toxicity of different DOPE–cationic lipid lipoplexes towards phagocytic macrophages and non-phagocytic T-lymphocytes. According to their results, this kind of cationic lipoplexes resulted to be extremely toxic following oral administrations. As a consequence, they claimed that alternatives to cationic liposomes for DNA therapy should be considered in order to avoid dose-limiting and adverse effects [11,12]. With the same spirit, Patil et al. studied delivery of plasmid DNA in mammalian cell cultures using anionic lipoplexes formed by mixtures of DOPE and anionic DOPG lipids [16]. According to their results, toxicity of lipoplexes prepared from naturally occurring anionic lipids revealed lower than the cationic liposome lipofectAmine®. Later, Lv et al. reported a very complete review with respect to the toxicity of cationic lipids and polymers in gene delivery in which a relationship of toxicity and structure of diverse cationic compounds was provided [17].

Recent investigations have revealed the complexation of anionic liposomes with DNA (anionic lipoplexes) as an alternative to classical cationic lipoplexes due to their lower cytotoxicity [16,22–37]. However, since both anionic lipids and DNA are negatively charged, cations are required as bridges between them. In this sense, monovalent cations are inappropriate due to their poor binding affinity with lipid headgroups [38]. Instead, multivalent metal cations, principally Ca^{2+}, have been employed to form anionic lipoplexes [16,22,23,26,27,29,32,37,39]. Precisely, the first section of the present manuscript is devoted to analyse in depth the experimental results concerning the complexation procedure of anionic lipoplexes as well as the interactions at the surface between anionic lipid monolayers and DNA mediated by cations. Given that, at some stage of the transfection process, the interfacial properties of these compounds are involved; their surface properties are of major importance for understanding the penetration (adsorption) mechanism of DNA and for identifying the conformational changes involved in the membrane. In this regard, Langmuir monolayers are a versatile and well established characterisation method which allows elucidating structural and mechanical properties of lipid surfaces. Furthermore, Langmuir monolayers serve as substrate to complementary techniques such as SPM [40], BAM [41], FI [42,43], IRRAS [41], VSFG [43], SHG [44], *etc*. These allow visualization of the interfacial material and provide further structural details rendering Langmuir monolayers a very attractive experimental method to study the interaction of DNA with lipids. The first part of this work reviews the current literature of Langmuir monolayers as a substrate for anionic lipid and DNA complexation.

In addition, state of the art of theoretical models and computer simulations related to the adsorption of DNA onto likely charged surfaces mediated by cations are also reviewed in this work. In particular, coarse grain (CG) models, in which several atoms are grouped together into effective interaction sites, appear as a satisfactory tool to study many phenomena that lie within the mesoscopic spatio-temporal scale. In a biological context, examples of such phenomena are protein–protein interactions, lipid–protein interactions, and membrane–membrane interactions [45]. In fact, CG molecular model implemented by MC simulations has been previously used to describe theoretical analysis of the physical properties of cationic and zwitterionic lipid–DNA complexes [46,47]. For the case of polyelectrolytes, Dias and Pais reported a complete review in which they discussed results from computer simulations

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based on CG polyion models applied to the conformation of polyanions as well as their condensation in bulk, induced by multivalent ions and oppositely charged polycations. They also studied the polyanion condensation in responsive charged surfaces. However, the case of adsorption of such polyions onto likely charged surfaces mediated by multivalent cations was not reported [48]. However, the case of adsorption of such polyions onto likely charged surfaces was not reported. Accordingly, in Section 3.1 we will use the works of Dias and Pais as a starting point to describe the CG models and afterwards we will present some preliminary results concerning adsorption of anionic polyelectrolytes onto anionic surfaces.

Beyond the CG models, conventional all-atomic Molecular Dynamics (MD) simulation (and hybrid simulation) is a well-established technique in order to simulate the structure and dynamics of soft matter in general, and of biomolecular systems in particular [49]. Approaches derived from CG models are correct in many situations but can become inaccurate for the so-called *last nanometer* phenomena [50]. These are interfacial processes with length scales of the order of the nanometer, whose description requires the inclusion of atomistic detail of the lipids. Examples of these phenomena are ion binding [51–54], membrane adhesion, sequestering of lipids by other biomolecules [55] and membrane crystallization [56]. Accordingly, the goal of the last section of the manuscript is twofold. Firstly, to understand in more depth the interaction of cations and lipids by means of all-atomic MD simulations and secondly, to include a DNA model in the simulations and examine its adsorption onto anionic lipid bilayers in the presence of the divalent cations. Alternatively to simulations, compaction of DNA by cationic liposomes has been also theoretically analysed by a phenomenological theory put forward by Nguyen and Shklovskii [57]. This theory provides phase diagrams that contain information about the liposome and DNA concentration necessary to have either anionic or cationic isolated lipoplexes or also clusters of lipoplexes [9,18,19]. Unfortunately, quantitative predictions for chemical contributions (hydrogen bonding, solvation effects,…) cannot be readily established from chemical structure or physical property considerations. The only direct way to estimate the strength of these chemical contributions seems to be from the adsorption behaviour at high salt concentrations, where the electrostatic contributions are gradually expected to become less important. Also, numerical calculations of the mean-field electrostatic free energy of a zwitterionic lipid monolayer–DNA complex support experimental observations where DNA adsorbs onto zwitterionic lipid monolayers in the presence of divalent cations [58,59]. These last results will be deeply reviewed in Section 3.

In accordance, this review provides an up to date list of references for this field of study and is organized into two sections concerning experimental and simulation studies of anionic lipoplexes, respectively. The first section is in turn divided in two subsections in terms of interactions of DNA with lipid bilayers and lipid monolayers. Regarding the modelling and simulation section, it has been divided according to the theoretical procedure in CG models and all-atomic MD simulations.

2. Experimental evidences

2.1. Anionic lipoplexes physiology

As it has been mentioned in the introduction, anionic lipoplexes become an alternative to classical cationic lipoplexes due to their lower cytotoxicity. In this sense, the group of Prof. Mozafari pioneered the use of anionic lipoplexes since the early 90s (see references [22,23,29–31,40,60] and references cited therein). This group has specialised in the preparation and optimisation of anionic lipoplexes reporting new strategies to incorporate polynucleotides to anionic liposomes by the mediation of divalent cations. For instance, they developed a method for transferring genomic DNA to target cells by means of liposomes formed by PC and DCP as zwitterionic and anionic lipids, respectively, and CHO. Likewise, they provided two possible transfection strategies. On the one hand, the procedure consists in the induction of fusion of liposomes (with DNA inside) through the catalytic action of DNA and Ca^{2+} in order to transfer the content of the liposomes into the target cell. On the other hand, the strategy was to introduce the DNA to a medium containing target cells and Ca^{2+} in such a way the DNA molecules can be taken into the fused liposomes [22,23]. In addition, Mozafari et al. have developed a scalable and safe method to prepare non-toxic anionic lipoplexes based on an original heating procedure so-called HM, in which no volatile organic solvent or detergent is used [30]. Therein, the authors indicated that HM-nanoliposomes were completely non-toxic in the cell-line tested, whereas conventional liposomes prepared by the usual thin-film method revealed significant levels of toxicity [30,60]. In addition, this group is skilled in the morphological characterisation of the structure of the resulting complexes by diverse techniques of microscopy [31,40]. As a consequence, the investigation performed by this group has inspired other researchers in the use of anionic lipoplexes for applications in gene therapy and thus, the number of experimental studies has grown vertiginously in the last decade. For instance, Patil et al. carried out studies concerning anionic lipoplexes for plasmid DNA delivery into mammalian cells *in-vitro* [16,26]. They reported a novel anionic lipoplex composed of DOPE/DOPG lipids, plasmid DNA molecules and Ca^{2+}. The resulting transfection and toxicity assays were evaluated in CHO-K1 cells achieving an efficient delivery of plasmid DNA with anionic lipoplexes [16]. In addition, these authors demonstrated that biophysical characterisation of anionic lipoplexes can be used to identify formulation conditions and electrochemical properties for achieving optimal transfections [26]. Afterwards, this idea was followed by Srinivasan et al. who also used DOPG/DOPE liposomes to study the role of Na^{+}, Mg^{2+} and Ca^{2+} at different lipid-DNA ratios to transfer plasmid DNA in CHO-K1 cells [33]. Hence, they carried out a biophysical characterisation of the resulting lipoplexes by using techniques of particle size, gel electrophoresis, transmission electron microscopy and confocal microscopy to optimise the formulation for these anionic lipoplexes. Interestingly, Srinivasan et al. demonstrated that the transfection efficiency of anionic lipoplexes is similar or higher than with cationic lipoplexes but the cellular toxicity was meaningfully less when anionic liposomes were used [33]. Continuing with the biophysical characterisation of DOPG/DOPE-DNA-Ca^{2+} lipoplexes studies performed by Patil et al., they used electrokinetic techniques to demonstrate that Ca^{2+} interaction with plasmid DNA also induces restructuring of DNA that facilitates the transfection [26]. Furthermore, they used circular dichroism and fluorescence spectroscopy techniques to show how a plasmid DNA underwent a conformational transition due to compaction and condensation upon Ca^{2+}-mediated complexation with anionic liposomes. In addition, Patil et al. proved that these structural changes were different to those found in cationic lipoplexes or in complexes DNA–Ca^{2+}. As a consequence they concluded that DNA cellular uptake, intracellular trafficking and transfection of anionic lipoplexes may involve different and more suitable pathways compared with other DNA delivery systems [26]. More recently, Kapoor et al., using DOPG/DOPE liposomes again, have demonstrated that physicochemical attributes of anionic lipoplexes can be used to estimate *in-vitro* activity of anionic lipid-based ternary siRNA complexes and understand their morphology correlating the lipoplex assemblies to biological activity [37]. Based on their experimental characterisation, they proposed a model representing lipid-siRNA association within the anionic lipoplexes as a function of the lipid-siRNA ratio that proves the existence of a synergism role of lipids and Ca^{2+} in associating with siRNA to form anionic lipoplexes. At low lipid-siRNA ratios, siRNA loading of anionic lipoplexes is equivalent to that obtained with Ca^{2+}-siRNA complexes (in absence of lipids). In contrast, at high lipid-siRNA ratios, Ca^{2+} primarily contributes to siRNA complexation whereas lipids contribute to siRNA encapsulation and

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both of these processes are required to achieve complete siRNA loading [37].

With the aim of further understanding the structural aspects of anionic lipoplexes, Liang et al. investigated the structure and interactions of anionic lipoplexes in the presence of different divalent metal cations such as Mg^{2+}, Ca^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+} and Zn^{2+} by performing SAXS experiments and confocal microscopy [25]. According to them, anionic lipoplexes self-assemble into a lamellar structure at low lipid membrane charge densities (Fig. 1 A). This lamellar assembly consisted in alternating layers of like-charged DNA and anionic membranes bound together with divalent cations. As the membrane charge density increased, they observed how the DNA was expelled from the complex and sandwich-like structure resulted among lipid membrane sheets and divalent cations (Fig. 1 B). This transition between the two structures was also induced by increasing the concentration of divalent cations. Moreover, the system forms an inverted hexagonal phase comprised of a hexagonal array of divalent cation-coated DNA strands wrapped by anionic lipid monolayers (Fig. 1 C). In addition, it was reported that divalent cations (especially Zn^{2+}) could also coordinate nonelectrostatically with lipid molecules and modify the resulting structure [25]. Later, Khiati et al. performed also SAXS studies of novel anionic nucleotide based lipids for DNA delivery and expression [32]. According to them, the structure of the complexes is strongly dependent on the formulation being the transfection enhanced by inverted hexagonal structures. In cooperation with the structural aspects of anionic lipoplexes, the equilibrium constants for the binary complexes Ca^{2+}–lipid, Ca^{2+}–DNA and DNA–lipid provide the basis for the electrotransfer of gene-DNA into biological cells and tissue [27].

Concerning the interaction between lipids and DNA, Michanek et al., studied the interaction of nucleic acids with zwitterionic and anionic phospholipids by means of DSC and QCM-D [61]. Therein the authors showed that the length of the nucleic acids is one of the crucial factors in determining how association influences the lipid phase behaviour, and the largest effects were observed for the shorter nucleic acids. Later the authors also characterised the association of ssRNA, dsRNA and dsDNA with zwitterionic and cationic monolayers with different phase behaviour by means of surface pressure–area isotherms, NR and fluorescence microscopy [62]. Also with cationic lipids, Suga et al., investigated the binding of liposomes to ssRNA molecules through electrostatic and hydrophobic attraction in order to induce conformational changes in them [63]. In the same line, binding of DNA to catanionic surfactant vesicles was also studied by Bonincontro et al. using different experimental approaches [64]. Therein it was shown how DNA can be completely retrieved from the vesicles upon addition of adequate amounts of anionic surfactant whereas precipitation of a poorly soluble salt results in an almost complete release of DNA.

In summary, experimental pieces of evidence of diverse anionic lipoplexes prove that the transfection efficiency of anionic complexes is similar or higher than that of cationic lipoplexes but the cellular toxicity is proved to be significantly less when anionic liposomes are used. The transfection efficiency of anionic lipoplexes depends on the optimal formulation of the complexes, which is a function, in turn, of the structure of the lipid–cation–DNA ternary system as well as the lipid–cation, cation–DNA and DNA–lipid binding. These experimental facts are summarised in Table 1.

2.2. Interaction of DNA with anionic lipid monolayers

The objective of investigating the interaction of DNA with anionic lipid by Langmuir monolayers is to enlighten the mechanism of formation of anionic lipoplexes and its transfection efficiency exposed above (Table 1). In order to address this issue effectively, separate investigations into the nature of the interactions between the various components involved in the complexation (DNA, lipid molecules and cations) should be accomplished. As it has been discussed, anionic lipids require multivalent cations to mediate attractions to negatively-charged DNA through direct electrostatic *bridging* interactions. But also, multivalent cations can coordinate non-electrostatically with lipid molecules and modify its structure and mechanical properties. Hence, it is important to start investigating the physics governing the effect of cations on the properties of anionic monolayers in order to address later the interaction of DNA with anionic lipid monolayers and achieve a coherent understanding of the resulting structures. Lipid monolayers at the air–water interface hence allow studying these interactions by looking into the modification of the area per lipid molecule and hence the compression state and molecular packing of the monolayer.

One of the first works dealing with anionic lipid monolayers was done back in the 80's by Ohki and Düzgünes [65] who use an original experimental approach which mimics the process of membrane fusion by looking into the adsorption of liposomes onto a monolayer comprised of the same lipids. These authors evaluate the surface tension of the system (PC and PS) as the liposomes penetrate into the monolayer as a function of concentration and type of the divalent cations present in the subphase (Ca^{2+}, Mg^{2+} and Mn^{2+}), thus examining the mechanism of the ion-induced membrane fusion in model membrane systems. As a result, the liposomes penetrate significantly into the monolayer only in the presence of a critical concentration of cations in the subphase and this mechanism is similar for both zwitterionic (PC) and anionic (PS) lipids. According to Ohki and Düzgünes, the interaction of divalent cations with the phospholipid polar group causes a water exclusion effect from the membrane resulting in an increased hydrophobicity of the cation–lipid complex. This is an important piece of work suggesting, for the first time, that the interaction between divalent cations and anionic lipid monolayers cannot be regarded as purely electrostatics but affects decisively the surface properties of the system. In fact, more recently it has been shown that the presence of Ca^{2+} or Mg^{2+} induces very different aggregation behaviour for PS liposomes as characterised by their fractal dimensions [66,67]. This ion-lipid specific

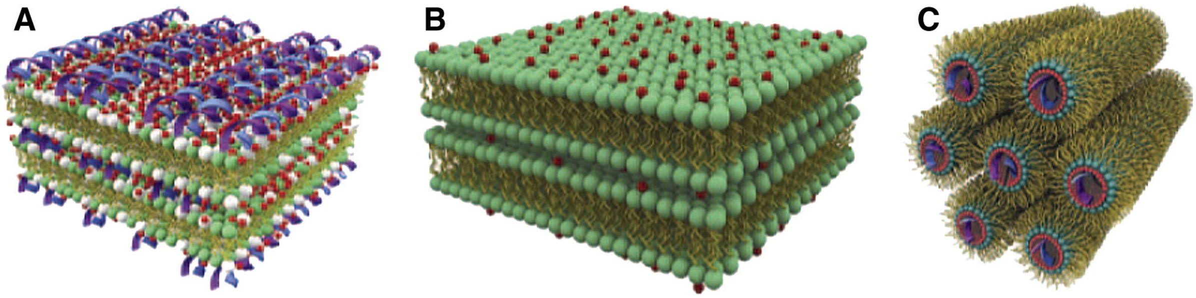

Fig. 1. Schematic pictures of self-organized structures of anionic lipoplexes mediated by divalent cations. (A) Condensed DNA–ion–membrane lamellar structure with alternating layers of DNA and anionic membranes glued together by divalent cations. (B) Condensed ion–membrane lamellar structure in which charged membranes stacks are held together by divalent cations. (C) 2D inverted hexagonal structure in which hexagonal arrays of divalent cations coated DNA strands wrapped in the anionic membrane monolayer tubes. Lipoplex model with DNA wrapped into phospholipid inverted micelles. Reprinted with permission from reference [25]. Copyright (2005) National Academy of Sciences, U.S.A.

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Table 1
Summary of experimental systems based on anionic lipoplexes reported in Sections 1 and 2.1.

Lipids	Lipid assemblies	Cations	Type of DNA-based therapeutic	Reference
N-C12-DOPE/DOPC (70:30)	liposomes	spermine^{4+}	plasmid DNA pZeoLacZ	[23]
DCP:PC:CHOL (2:7:1)	MLV	Ca^{2+}	genomic DNA from wheat seedlings	[21,22]
DCP/PC/CHOL (2:7:1)	MLV	Ca^{2+}	plasmid DNA pCMV-GFP	[29]
DCP/PC/CHOL (2:7:1)	MLV	Ca^{2+}	plasmid DNA pcDNA3.1/His B/lacZ	[30]
DOPG/DOPE (17:83)	MLV	Ca^{2+}	plasmid DNA pCMV-GFP	[15,25]
DOPG/DOPE (1:1-1:9)	MLV	Ca^{2+}, Mg^{2+}, Na^{+}	plasmid DNA pEGFP-N3	[32]
DOPG/DOPE (variable)	ULV	Ca^{2+}	siRNA	[36]
DOPG/DOPC (variable)	MLV	Mg^{2+}, Ca^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+}, Zn^{2+}	λ-phage DNA calf timus DNA	[24]
DOPG/DOPE DPPA/DOPE (variable)	Liposomes	Ca^{2+}	plasmid DNA pEGFP	[31]
PS/POPC (1:2)	ULV	Ca^{2+}	calf timus DNA	[26]
POPA	Liposomes	–	polynucleotide acid (polyU)	[27]
POPG, POPN	Liposomes	–	polynucleotide acids (polyA, polyU)	[33]
DMPS/DMPC (1:2)	MLV	Na^{+}, Ca^{2+}	tRNA and ssDNA	[61]

interaction has also been intensely studied by MD simulations for the case of Ca^{2+}, Mg^{2+} and La^{3+} cations interacting with PS lipid bilayers [52,53] and will be properly discussed in the last section.

More recently, Sovago et al. authored a very interesting contribution addressing specifically the interaction of Ca^{2+} with anionic lipid DOPS and zwitterionic lipid DPPC by means of Langmuir monolayers in combination with VSFG. This work emphasizes the role of the surface pressure on the calcium-induced changes of the lipid monolayer. Namely, Ca^{2+} induces the formation of lipid domains at low surface pressure (<5 mN/m), induces disorder at intermediate surface pressures (5–25 mN/m) and expands and simultaneously orders the lipid chains at the highest surface pressures (>25 mN/m) [43]. Interestingly, the authors also report qualitatively similar effects on anionic and zwitterionic lipids. Hence, this work confirms the importance of solvent interactions in the systems envisaged by Ohki and Düzgünes [65].

The influence of the surface pressure on the interaction of cations with anionic lipids has been also reported for an organic cation (MG) and DPPG [44]. Kim et al. show again distinct types of adsorption of MG depending on the initial phase of the monolayer. Combination with second harmonic generation, fluorescence microscopy and X-ray reflectivity illustrates the orientation of MG in each phase: vertical penetration into the DPPG monolayer at a fluid phase; vertically but oppositely paired orientations at a more condensed phase and parallel adsorption at the interface near the lipid headgroup region at a highly packed condensed phase.

More recently Ross et al. visualized the calcium induced domains in mixed DPPC/DPPS 4:1 Langmuir-Blodgett monolayers [42]. By means of a combination of imaging techniques, the authors demonstrate that these lipids are miscible in the absence of Ca^{2+} while they form DPPS rich domains upon the addition of Ca^{2+}. This study concentrates on low-intermediate surface pressure values and concludes that the presence of Ca^{2+} triggers phase separation of the monolayer [42]. In addition, the presence of Ca^{2+} was reported to alter the morphology of the domains in a DPPC/DOPS bilayer [68].

Hence, analysis in conjunction of the studies published so far concerning the effect of cations on anionic lipid monolayers allows establishing some details of the possible scenario for the complexation. Apart from the electrostatics, the presence of cations affects crucially the hydrophobicity of the system and the molecular packing of the monolayer. However, experimental investigations remain to be done in order to fully elucidate the role of the structural position of the anionic group in the lipid molecule and the role of the molecular packing of the lipid in the monolayer in the interaction of lipid with cations and the induced morphological changes in the lipid surface. Yet, the surface pressure seems to play a vital role in the cation–lipid interactions allowing the tuning of the molecular packing on the monolayer.

Consider now the interaction of DNA with anionic lipids. Since anionic lipids and DNA are electrically repelled to each other, the attraction needs to be mediated by multivalent cations, and this is the reason why the physical state of the lipid–cation monolayer has been established first. Nevertheless, none of the works shown so far apply the findings to then allocate DNA in the system. There is indeed very little work dealing with the interaction between DNA and anionic monolayers and the research in this direction remains in a very early stage.

There are some works addressing the complexation of DNA with zwitterionic lipids monolayers mediated by cations [41]. Interestingly, and in the line of the previous speculations, Gromelski et al. reported that the interaction with DNA is not only mediated by cations but also depends on the surface pressure of the lipid monolayer. These authors showed that the DNA partially penetrates into the DMPE monolayer only at low surface pressures and in the presence of Ca^{2+} but is squeezed out at higher surface pressures even in the presence of Ca^{2+}. This again demonstrates the importance of the physical state of the lipid monolayer for establishing the conditions which favour the complexation of DNA with the lipid. The presence of Ca^{2+} is not enough to trigger not only the complexation but also the molecular packing (*i.e.* surface tension) needs to be considered.

One of the few works showing specifically adsorption of DNA onto anionic lipid monolayers is the work done by Frantescu et al. with PS/POPC 1:2 [27]. These authors report an increase of the area per lipid in the presence of DNA + Ca^{2+} in the subphase. In this way, the authors provide evidence of the binding of DNA to anionic lipids mediated by Ca^{2+}. Interestingly, in contrast to DMPE monolayers [41], the DNA complexation with PS/POPC 1:2 mediated by Ca^{2+} remains regardless of the surface coverage of the monolayer. Whereas DNA is expelled in DMPE monolayer at high surface pressures, for PS/POPC the lipid remains in a liquid-expanded state during all the compression. Hence, an important conclusion arising from these studies is that unsaturated chain phospholipids which reach the collapse directly from liquid-expanded

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phase never reach enough condensation to squeeze out the DNA in the presence of Ca^{2+}.

With the aim of deepen into this effect, a recent study carried out at the University of Granada deals with mixtures of zwitterionic and anionic lipid but increasing the concentration of the former (DPPC/DPPS 4:1) [69]. Fig. 2 shows the shift in the π-A isotherms recorded for a DPPC/DPPS 4:1 monolayer on a subphase containing both Ca^{2+} and DNA with respect to the one recorded on a water subphase [69]. Interestingly, the penetration now depends again on the surface pressure with the isotherms appearing displaced to higher molecular areas only at low surface pressure while collapsing at the highest surface coverage. This result agrees with the results of Gromelski et al. for DMPE [41], suggesting that the highest molecular packing reached before the collapse for saturated phospholipids forces the DNA out the monolayer. The highest amount of zwitterionic lipid in the system could be now dominating the interaction. Fig. 3 shows AFM images of the monolayer of both isotherms at $\pi = 2$ mN/m. These images reveal the presence of DNA at the interface, allowed identifying phospholipid–Ca^{2+}–DNA interfacial aggregates and picture more accurately the interaction [69]. Moreover, visualization of the monolayer by AFM has provided the definite evidence of the presence of DNA at the interface.

Results published so far importantly highlight the different interactions occurring in zwitterionic and anionic monolayers and we are beginning to understand how they influence the complexation with DNA mediated by Ca^{2+}. Experimental works with Langmuir monolayers demonstrate not only the importance of cations in mediating the interaction between anionic lipids and DNA but also reveal the role of the nature of lipid and molecular packing as quantified by surface tension. The adsorption of DNA appears more stable for anionic systems but further investigation into this mechanism still needs to be undertaken in order to fully provide a clear explanation of the interactions taking place and validate this as a universal trend. To achieve this it seems crucial to combine it with more sophisticated techniques in order to univocally interpret the results. Furthermore, work on bilayers and numerical simulations (as those reviewed in next sections) need to be considered in order to realize the mechanism of nucleic acid transfer by liposome–DNA complexes.

3. Modelling and simulations

Before analysing the mechanism underlying the formation of anionic lipoplexes, modelling the system zwitterionic monolayer/M^{2+}/DNA is essential to understand the role of divalent metal cations in its interactions with zwitterionic lipids and DNA. The formulation of a general theory about the DNA adsorption onto zwitterionic monolayers in the presence of divalent cations would be helpful to correlate such results, determine the driving force and the conditions that favour the formation of the monolayer/M^{2+}/DNA complexes and describe their structural features. In this sense, Mengistu et al. have developed a theoretical approach based on the classical PB formalism to look into the role of divalent cations in the formation of a monolayer/M^{2+}/DNA complex [58]. To this aim, they represented the DNA with charged rods. The zwitterionic headgroups in the monolayer were modelled as electric dipoles. The dipole movement was limited by two constraints: i) The negative charge was fixed on the interface plane; and ii) the distance between the positive and negative charges was constant. They calculated the increment in the free energy associated with the adsorption of the DNA rod onto the zwitterionic monolayer, showing that this magnitude decreased with increasing concentrations of either monovalent or divalent cations, and being more sensitive to the divalent ones. Hence, the addition of divalent cations made the DNA binding on the zwitterionic monolayer more stable. Moreover, the adsorption process took place with redistribution of divalent cations from the DNA to the anionic moieties in the lipid headgroups. Consequently, the formation of the monolayer/M^{2+}/DNA complex seems to be governed by the electrostatic interactions. This model was recently extended to account for the change in the surface pressure induced by the M^{2+}-mediated DNA adsorption on the zwitterionic monolayer [59]. In this case, the authors focused on the second order tilt transition, which is observed experimentally at lower surface pressures when both M^{2+} and DNA are present in the subphase [41]. This downshift in the tilt transition surface pressure was qualitatively predicted by the model, indicating that the DNA-induced M^{2+} penetration into the headgroup region resulted in a lateral condensation in the monolayer. Although this theory explains satisfactorily the M^{2+}-mediated DNA adsorption onto zwitterionic monolayers, the implications of the introduction of an excess of negatively charged moieties in the monolayer have not been analysed yet. Undoubtedly this feature deserves a great attention in the future since physical–chemical properties are probed to be significant on the transfection activity of cationic lipoplexes [70].

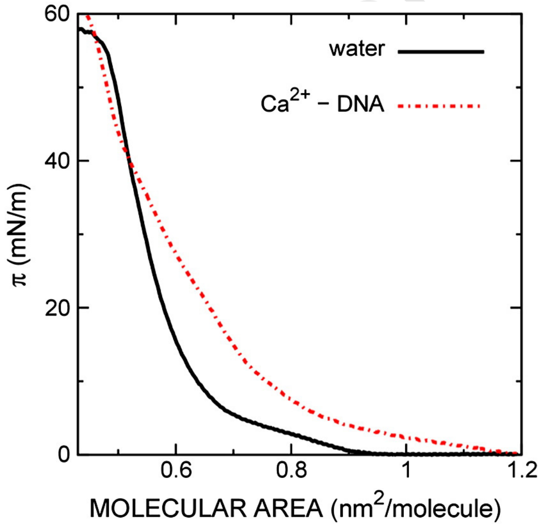

Fig. 2. DPPC/DPPS 4:1 surface pressure–area isotherms on a pure water subphase (black, solid) and on a solution containing both DNA and Ca^{2+} (red, dash-dot). More details in reference [69]. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

The interactions of DNA with a negatively charged surface are not intuitive and have been therefore less studied. Waltkins et al. modelled ssDNA tethered to a hydroxylalkane-coated gold substrate, which is negatively charged in aqueous media [71]. To determine both the DNA conformation and the adsorption on the surface at different salt concentrations, they balanced the contributions to the folding free energy due to electrostatic interactions and excluded volume effects as well, concluding that the surface attachment stabilized the folded state respect to the unfolded state at high ionic strengths. In addition, the work of Waltkins et al. demonstrates that the presence of cations can govern the DNA interfacial adsorption on negatively charged surfaces. In any case, the systematic search for the conditions in which metal cation mediated DNA binding to anionic monolayers can be appropriately addressed with simulation methods. The advantage of this approach is the possibility to test a wide range of cases by varying parameters such as the surface charge density, the length and geometry of the DNA and the valence and concentration of the metal cations. In addition, simulations can easily account for ionic correlations neglected by classical approaches (such as the PB theory).

The accuracy of a certain simulation to predict the mechanism of the DNA adsorption to a charged plane and the structural features of the monolayer/M^{2+}/DNA complex depends strongly on the model chosen for each of the different components of this system and the interactions applied among them. Accordingly, two well-differenced sorts of models can be used: CG models, which imply a simplification of the charged objects involved (polyelectrolyte, surface and ions), and all atomistic models, which take into account the interaction among all the atoms in the system, including water molecules. The former is ideal to explore

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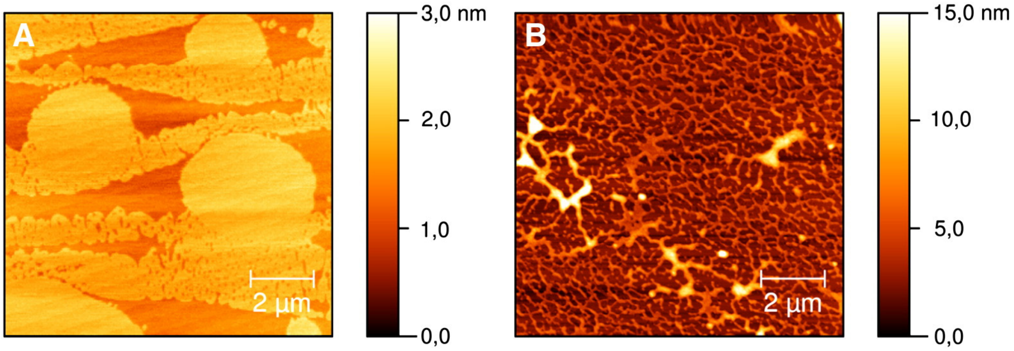

Fig. 3. AFM images of DPPC/DPPS 4:1 Langmuir Blodgett monolayers transferred from a pure water subphase (A) and from a solution containing both DNA and Ca^{2+} (B). More details in reference [69].

a vast range of conditions in a reasonable time and the latter offers us better precision, structural and conformational information and the effect of the solvent too, but the computational cost is much higher. Although both models provide valuable information, we cannot forget that they are approximations that could not reproduce reality perfectly. On one hand, a large number of effects derived from the molecular nature of the solvent and macromolecules are not included in CG models. On the other hand, all-atomic models consider all the atoms present in the relevant chemical species and include also their bonds, however chemical reactions, titration, electron transfer and in general processes which require a quantum description are typically ignored. At this point, it is important to remark also that both procedures consider model systems for the charged interface which are far from the complexity of a real cell membrane. For instance, homogeneous membranes composed only by phospholipids are considered in all-atomic models whereas real membranes have also several types of proteins and also protein channels. Accordingly many of the phenomena that take place in the cell membrane cannot be properly studied by using the cited models. In addition, the effects derived from a global curvature of the mono/bilayer have not specifically studied in the present work. Undoubtedly this feature is an interesting subject with important implications since the elastic bending constants of lipid assemblies determine a variety of membrane-associated physical and functional properties such as the membrane vesicle shape, the structure and formation of interlamellar attachments and non-lamellar lipid phases, and may also play an important role in membrane fusion [72].

Once the models are proposed, a suitable methodology to solve them is required. One possibility is to use approximate theories or to employ simulation techniques which are, in principle, exact [73]. According with our previous experience, we have chosen to consider MC and all-atomic MD simulations to solve the CG and all-atomic models, respectively. Hypothesis and approximations associated to each simulation method are described in the next sections. In particular, we first describe the basis and performance of some CG models below whereas all the atomistic simulations are treated in the next section.

3.1. Coarse grain models

In many cases, CG models consist in a bead-spring representation where hard charged spheres are used to build the system to be simulated. For instance, the salt ions are represented by spheres with the same electric charge and the equivalent radius. The electrostatic interactions are usually computed by using a Coulomb potential:

$$u_{12}(\mathrm{elec}) = \frac{q_1 q_2}{4\pi\varepsilon_0\varepsilon_r r_{12}}, \quad r_{12} \geq R_1 + R_2 \qquad (1)$$

where q_1 and q_2 are the electric charges of the ions 1 and 2 respectively, R_1 and R_2 are their radius, r_{12} is the distance between them, ε_0 is the vacuum electric permittivity and ε_r the relative electric permittivity of the solution. All the magnitudes are expressed in the International System. Usually, Eq. (1) is expressed as:

$$u_{12}(\mathrm{elec}) = \frac{z_1 z_2 k_B T \lambda_B}{r_{12}}, \quad r_{12} \geq R_1 + R_2 \qquad (2)$$

where z_1 and z_2 are the valences of the particles 1 and 2 respectively, k_B is the Boltzmann constant, T is the absolute temperature and λ_B is the Bjerrum length, which is defined as the distance between two monovalent particles at which the pairwise electrostatic potential is equal to the thermal energy ($k_B T$):

$$\lambda_B = \frac{e^2}{4\pi\varepsilon_0\varepsilon_r k_B T} \qquad (3)$$

where e is the elemental charge.

Ions are allowed to move freely throughout the simulation cell, and excluded volume effects are usually introduced with either a hard sphere term or a LJ term (particularly when derivatives of the interaction energy are required):

$$u_{12}(\text{hard-sphere}) = \infty, \quad r_{12} \leq R_1 + R_2 \qquad (4)$$

$$u_{12}(LJ) = \left\{ \begin{array}{ll} 4\epsilon\left[\left(\frac{d}{r}\right)^{12} - \left(\frac{d}{r}\right)^{6} + \frac{1}{4}\right], & r \leq r_c \\ 0 \quad , & r > r_c \end{array} \right\} \qquad (5)$$

where is the depth of the potential well, $d = R_1 + R_2$. The LJ potential is cut-off at $r_c = 2^{1/6} d$ yielding a purely repulsive interaction.

Potentials described with Eqs (1), (2), (4) and (5) are applicable not only to the salt ions, but also to all sorts of beads present in the system.

The negative net charge is introduced in one face in the simulation cell to mimic the headgroup side of the lipid monolayer. However, a plane with a surface charge density does not take into account the properties of corrugation, discrete charges and the presence of both negatively and positively charged groups on the interface. This is the reason why the charge is usually introduced with charged beads embedded in the wall [74]. The sign of the charge of each embedded bead can be positive or negative but the number of the latter must be greater than the former to ensure that the plane has a negative net charge. In addition, these embedded beads can be fixed or they move along the plane to model the effect of the lateral diffusion.

The last item of the model to be described is the DNA. In a CG representation, its structure is usually simplified as a flexible chain of negatively charged beads. The diameter and the equilibrium separation distance between two adjacent beads in the DNA string slightly depend on the sort of interaction that is defined to keep the string cohesion. According to the literature reviewed, two different sorts of bond potentials can be applied. On the one

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hand, the elastic bond potential connects each pair of adjacent beads by a spring [48,75,76]:

$$u_{elastic}\left(r_{n-1,n}\right) = \frac{1}{2}k\left(r_{n-1,n} - r_{eq}\right)^2 \tag{6}$$

where k is the bond constant, $r_{n-1,n}$ is the distance between the two adjacent beads in the DNA string $n-1$ and n and r_{eq} is the equilibrium distance between the two adjacent beads. k and r_{eq} can be adjusted to match the DNA bond properties. All the magnitudes are expressed in the International System.

On the other hand, when the DNA polyelectrolyte is described as a sequence of beads connected with nonlinear springs, a FENE potential is applied [77]:

$$u_{FENE}\left(r_{n-1,n}\right) = \frac{1}{2}k' r_0^2 \ln\left(1 - \frac{r_{n-1,n}^2}{r_0^2}\right) \tag{7}$$

where k' is the bond constant and r_0 is the maximum bond distance. k' and r_0 can be adjusted to match the DNA bond properties. All the magnitudes are expressed in the International System.

Similarly, the flexibility properties of the DNA are modulated by an angular potential:

$$u_{angular}\left(\alpha_{n-1,n,n+1}\right) = \frac{1}{2}k_{ang}\left(\alpha_{n-1,n,n+1} - \alpha_{eq}\right)^2 \tag{8}$$

where k_{ang} is the bending constant, $\alpha_{n-1,n,n+1}$ is the angle formed by the three adjacent beads in the DNA string $n-1$, n and $n+1$ and α_{eq} is the equilibrium angle between the three adjacent monomers. Again, k_{ang} and α_{eq} can be adjusted to match the DNA bending properties. All the magnitudes are expressed in the International System.

Dias et al. applied the CG model to search for the conditions that lead to a negatively charged polyelectrolyte to condensate under different geometries [48]. They run MC simulations to separately study, on the one hand, the polyelectrolyte condensation by metal cations in bulk and, on the other hand, the binding on a charged surface without any added salt. Although these cases cannot represent the formation of the anionic monolayer/M^{2+}/DNA, they should be considered as important precedents because the simulation methods and the factors governing condensation described in their works may inspire future computational research on the anionic monolayer/M^{2+}/DNA system. This is the reason why we would like to review the most remarkable findings reported by these authors about the effect of lateral diffusion and the polyelectrolyte geometry on the adsorption on a charged surface.

Lateral diffusion in lipid monolayers that contain both positively and negatively charged headgroups make them to behave as responsive surfaces, since they undergo a reorganization of the surface charge to accommodate the polyelectrolyte. In fact, mobile charged bead embedded surfaces adsorb a higher number of monomers from both flexible and semiflexible polyelectrolytes than fixed ones independently on the surface net charge [76]. This finding suggests that the membrane fluidity could be essential for the mechanisms of formation of lipoplex and transfection efficiency. In addition, the same authors reported recently differences in interfacial adsorption due to circular or linear polyelectrolytes [75]; the former adsorbed more strongly on both fluid and frozen interfaces with respect to the latter. This effect was stronger for polyelectrolyte binding onto weakly and positively charged surfaces. However, when the fluid surfaces lacked of net charge the circular polyelectrolyte was totally desorbed while the linear one retained a certain degree of adsorption. This difference between the simulation results observed for linear and circular polyelectrolytes points out that, when electrostatic interactions are low enough, the polyelectrolyte-responsive surface interaction is governed by a delicate balance between the entropy of the surface groups and that of the polyelectrolyte.

It should be mentioned, however, that the surveys about adsorption on responsive surfaces published by Dias et al. were carried out exclusively in the presence of monovalent counterions. In addition, they only considered the minimum number of counterions required for electroneutrality. For monolayer/M^{2+}/DNA complexes, however, it would be desirable to account for the presence of multivalent cations and anions.

Similar bead-spring models have also been applied to study the DNA properties in solution with MD simulations [77–81] and some variations have been proposed to address the problem with different geometries. Since lipoplexes are bulk systems formed as a result of the interaction of DNA with liposomes, which are spherical colloids, models of charged curved surfaces are likely to be helpful in lipoplex modelling. This challenge has been tackled by Carnal et al [82]. They have run MC simulations with one negatively charged polyelectrolyte condensing on a positively charged spherical nanoparticle. They were able to determine the influence of parameters affecting the adsorption, such as the surface charge density of the nanoparticle, the valence of the salt and the pH, but nanoparticles with negative net charge were not considered. This work could be applicable to the cationic lipoplex early stage formation modelling and could be modified to study the interaction anionic or zwitterionic liposome/M^{2+}/DNA.

CG approaches can be formulated to predict the structure of lipoplexes. For instance, Farago et al. proved that GC models can capture the self-assembly of cationic lipid–DNA complexes [46,47]. In fact, they found excellent agreement with x-ray diffraction experimental data for the dependence of the spacing between DNA chains on the cationic lipid concentration. At high charge densities, they observed that the increasing electrostatic pressure exerted on the membranes leads to pore opening, through which the DNA may be released from the complex. Also, zwitterionic phospholipid/M^{2+}/DNA self-assembly has been observed *in silico* at different length scales. On the one hand, at short range, Tresset et al. performed MC simulations to gain insight into the effect of different cations in the formation of lipoplexes from zwitterionic phospholipids [83,84]. In this case, a homogeneously charged rod represented DNA, while phospholipid molecules were modelled with three beads: two of them lacked electric charge and represented the hydrophobic tail and the other one held an electric dipolar moment and represented the zwitterionic headgroup. At equilibrium, the phospholipid molecules formed inverted micelles, being the multivalent cations placed in the space between the DNA rod and the phospholipid headgroups, as has been experimentally measured by SAXS. On the other hand, more recently, the same author proposed these inverted micelles as building blocks to simulate the structure of the assemblies at the supramolecular level [85,86]. In this manner, such inverted micelles are now modelled as semiflexible tubes interacting with one another through electrostatic interactions, hydrophobic force and excluded-volume repulsion. As a result, the tubes formed clusters whose morphology depended on size and rigidity of the tube. In solution, the cluster should be wrapped into a lipid monolayer as shown in Fig. 5, because the polar headgroup contact with the water molecules should be more favourable. To sum up, different simulation methods based on CG models comprise a powerful tool to deduce the structural properties of lipoplexes. However, the effect of anionic lipids alone or in combination with zwitterionic ones is still to be elucidated.

In fact, only a few CG simulations have explicitly studied a related problem: the complexation of negatively charged polyelectrolyte with a like-charged surface. Messina et al. analysed the complexation of a highly charged sphere with a flexible polyelectrolyte, both negatively charged, in the presence of divalent and trivalent counterions but in salt-free environment [87]. For typical electrostatic couplings of aqueous solution, a weak like-charge complexation takes place provided that the linear charge density or the counterion valence is high enough. More recently, Turesson et al. have investigated a similar issue: The calcium mediated polyelectrolyte adsorption on a like-charged surface [88]. Surface charge was uniformly smeared. They conclude that the purely electrostatic adsorption is also weak, but can be considerably increased when ion pairing effects between calcium and carboxylate

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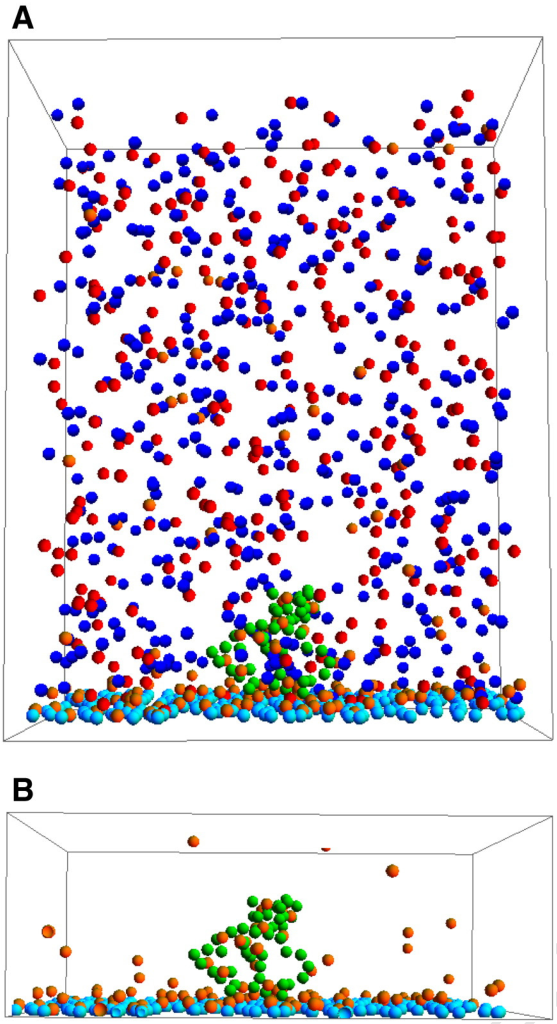

Fig. 4. A) Snapshot showing a negatively charged polyelectrolyte chain (green beads) in the vicinity of a negatively charged surface (light blue beads). Orange, red and dark blue spheres represent multivalent cations, monovalent cations and monovalent anions. B) This snapshot shows only the charged surface, the polyelectrolyte chain and the multivalent cations near the surface for the sake of clarity. As can be seen, a few multivalent cations behave as a bridge between the polyelectrolyte chain and the surface, bringing them together (in spite of the fact that they are likely charged). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

monomers are taken into account. From density functional theory calculations, Wang et al. have also reported multivalent counterion mediated polytelectrolyte adsorption onto a like-charged surface. In this case, however, they only consider purely electrostatic correlations [89].

It would be interesting to extend the previous works to responsive surfaces. In this sense, we have included in this review our preliminary study of the adsorption of a negatively charged polyelectrolyte onto a negatively charged responsive surface in the presence of a 3:1 electrolyte (trivalent cations, monovalent anions). A flexible 50-monomer polyelectrolyte chain (the charge of each monomer is $-e$) identical to that investigated by Dias et al. has been considered. However, the responsive surface is now exclusively made of negative mobile groups and is strongly charged (-0.16 Cm^{-2}). The electrolyte concentration is 50 mM. The dimensions of the simulation cell are 15 nm × 15 nm × 20 nm. The long-range electrostatic interactions were handled using the method proposed by Boda et al. for slab geometry (periodicity only in two dimensions) [90]. This simulation suggests that the polyelectrolyte chain can stay in the neighbourhood of the likely charged surface due to the role of multivalent counterions (see Fig. 4). It is well known that these ions form a layer on the surface that neutralizes its charge to a great extent and might even overcharge it. In addition, they would also screen the charge of the polyelectrolyte chain, which could wander in the vicinity of the charged surface without being strongly repelled. Fig. 4 also suggests that, eventually, some beads of the polyelectrolyte chain could be adsorbed onto such multivalent cations.

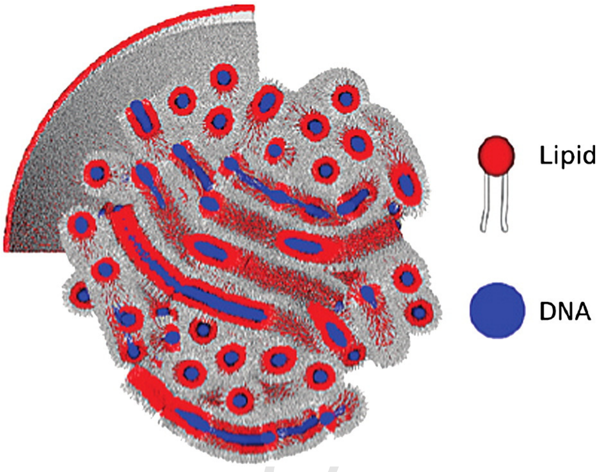

Fig. 5. Lipoplex model with DNA wrapped into phospholipid inverted micelles. Reprinted with permission from reference [85]. Copyright (2011) American Chemical Society.

3.2. All-atomic molecular-dynamics simulations

3.2.1. Cation–lipid interactions in lipid bilayers

As was previously pointed out, the standard view of the interaction of electrolyte with interfaces is based on a simplified picture of the interface as a simple geometrical boundary with uniform charge (typically a charged plane) [91]. This standard view can be implemented for example by considering the PB classical theory of double layer electrostatics, usually employed not only for colloids but also for soft-matter systems and biomolecules in general. As long as the limitations of the PB theory become clear, more sophisticated theoretical approaches focused on the refinement of the description of the ions (ion size, polarizability,…) but retained an extremely simplified description of the interface as a boundary condition. In the case of complex systems such as lipids membranes, interactions are highly specific, *i.e.* depend strongly on the particular lipids and ions involved. Hence, at this scale, the hydration behaviour of ions plays a major role, even for multivalent cations [51–53], in which interface–ion interaction was supposed to be mainly electrostatic. For example, experimental evidence (from turbidity measurements and other techniques) shows that similar divalent cations such as Ca^{2+} and Mg^{2+} induce different structural changes in phospholipid membranes and different membrane solvation, which in turn has substantial consequences in the interaction between membranes and their aggregation behaviour [53,65–67]. Experimentally, it is also known that the binding of multivalent cations to negatively charged lipids is endothermic, and thus it is entropy driven [92,93]. This fact strongly suggests the existence of hydration and solvation effects as well as structural rearrangements in the membrane as the driving forces for ion binding, effects which cannot be captured by CG models.

The question is how to study theoretically all this complexity, relevant at the last nanometer scale near a lipid assembly. The continuous increase in computer power and the development of new algorithms for large-scale MD simulations has made possible its use as a kind of computational microscope to study molecular systems and processes with atomic resolution. The development of force fields for lipids has allowed large scale atomistic simulations of lipid assemblies and their interactions with unprecedented resolution. Conceptually, the MD

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method is based on the fact that we know the basic equations governing the motion of atoms and molecules, so we can employ numerical solutions of these equations to predict the behaviour and properties of matter from their molecular composition [91]. As an essential input, the MD technique requires a molecular model of the individual entities building up the system (all atoms and bonds making up the molecules and a prescription for calculating atom–atom interactions). As an output, MD simulations provide equilibrium and transport properties of the system. More importantly, MD simulations give a detailed account of the different molecular contributions to the properties of the system and allow one to identify the molecular origin of the observed behaviour. Of course, as any approach to a difficult problem, these simulations have their own difficulties. MD simulations of realistic models of lipid systems (with accurate atomic resolution) are far from easy. Also, the need for simulating large numbers of water molecules (in order to describe both interfacial phenomena but also bulk water and electrolyte) leads to the requirement of large amounts of computational power, which typically require the use of supercomputing facilities. In any case, MD results offer us many important insights which should be considered and incorporated in more general theories.

As an illustration of the kind of results, typical of MD simulations of ions and lipids, we show in Fig. 6 a snapshot of a simulation of a PS membrane in the presence of divalent counterions Ca^{2+} or Mg^{2+} [53]. As illustrated in this snapshot, the binding of a single Ca^{2+} cation typically involves a −COO group from one PS lipid and a PO_4^- group from another lipid. On the other hand, Mg^{2+} has typically two ways of binding to PS lipids. One is rather superficial, which involves two −COO groups from two different PS lipids. Mg^{2+} cations bound in this way remain attached to the exterior of the membrane, but are not incorporated inside the hydrophilic part of the membrane. The other binding mode involves the binding of Mg^{2+} with two phosphate groups. This binding mode (which is less probable) implies a deeper penetration of Mg^{2+} than Ca^{2+}. Although Ca^{2+} and Mg^{2+} cations have similar affinities to PS lipids, they bind in a very different way and induce a different structure in the lipid membrane. Also, the hydration involved in the binding of these ions is different. MD simulations predict that PS membranes are more hydrated in the presence of Mg^{2+}, in agreement with FTIR experiments [94]. In the simulations of reference [53], it was obtained 7.5 water/lipid(Ca^{2+}) as compared with 9 water/lipid(Mg^{2+}).

The case of the interaction of trivalent ions with anionic lipids is also interesting and provides interesting clues on the mechanism of lipid–ion interaction. It has been shown that La^{3+} is able to induce charge inversion in PS liposomes, at very low concentrations of the order of $c_0 \approx 0.1$ mM [52]. This means that at concentrations larger than c_0, the electrokinetic charge of the PS liposome is positive, instead of negative. The positive charge from the adsorbed ions is larger than the bare (negative) charge from the lipids. This charge inversion effect is well known in many systems (including latex colloids [95–99], silica [100], peptides [101] and many other systems). However, in the case of lipid systems it has an important peculiarity. In many of these examples, the addition of physiological concentrations of monovalent electrolyte suppresses the observed charge inversion but in the case of lipids, the addition of monovalent electrolyte has no effect in the charge inversion concentration [52]. Theoretical considerations show that monovalent salt is able to weaken or even destroy the ionic correlations responsible for charge inversion in materials like silica [100] and latex [102]. In the case of lipids, a different mechanism, robust against the screening by physiological amounts of 1:1 salt, is operating [51,52]. According to MD simulations [52], La^{3+} penetrates deeply into the hydrophilic region of the PS membrane, at a position equivalent to that of Ca^{2+} cations. At that position, La^{3+} cations become associated to about 5 oxygen atoms from the PS lipids, larger than the value found for Ca^{2+} (4 oxygen atoms) or Mg^{2+} (3 oxygen atoms). This direct interaction between oxygen atoms of PS and Ca^{2+} inside the hydrophilic region of the membrane is well "protected" against screening by monovalent salt (Na^+, NO_3^-, in this case) which remains in solution. Of course, there is some competition between La^{3+} and Na^+ for binding to oxygen atoms of PS, but the affinities are rather different. The estimated free energy of binding per La^{3+} is about $-9\ k_BT$, much larger than the $-3.5\ k_BT$ estimated for Na^+.

These studies also demonstrate another interesting feature of the lipid–ion interaction. Both theories and many experiments support the concept that an increase of the surface charge density enhances the interaction between ions and the surface, for obvious electrostatic reasons [103]. However, in the case of the interaction between ions and lipids, this is not the case. For example, the free energy of interaction between La^{3+} and zwitterionic PC lipids is about $-12\ k_BT$, larger than the free energy for the interaction of La^{3+} with anionic PS quoted above [52]. The interaction of the cations with lipids is governed by the chemical structure of the lipids and by the particular location (accessibility, flexibility…) of negatively charged groups and not by the bare charge of an idealised surface.

3.2.2. DNA adsorption onto anionic lipid bilayers induced by multivalent cations

As it has been mentioned along the manuscript, the formation of anionic lipoplexes is achieved by the creation of a ternary complex between DNA and anionic liposomes using multivalent ion bridges. This feature has been shown in the numerous experimental evidences cited above as well as in the simulations presented in the previous section. Furthermore, the restructuration procedure of the lipid membranes induced by the multivalent cations is also analysed in the previous section. However, the DNA adsorption onto an anionic lipid membrane mediated by cations at the molecular level has not been described yet. To this end, we have performed preliminary all-atomic MD simulations about the adsorption of DNA onto a PS bilayer in the presence of Ca^{2+}.

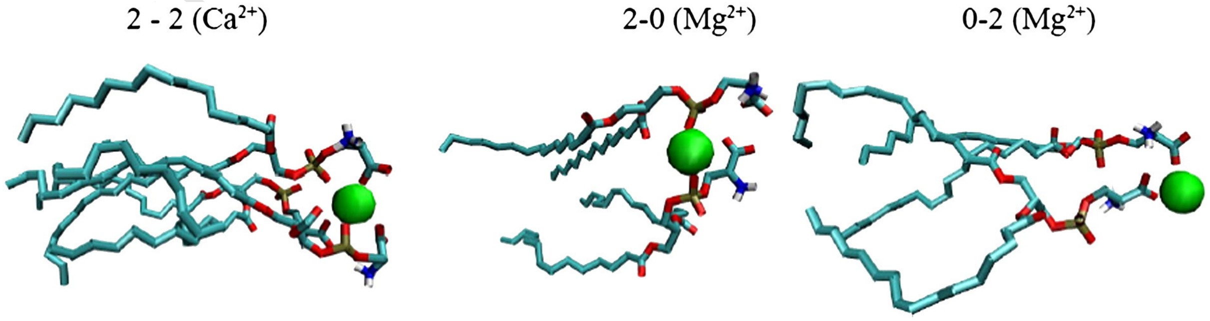

Fig. 6. Caption corresponding to the binding of Ca^{2+} or Mg^{2+} cations to PS lipids as obtained in MD simulations from reference [53]. The lipids are shown as lines (the colour code of each atom follows the standard crystallographic convention) and the cations are shown as green spheres. We show the most probable binding mode for Ca^{2+} (left) and the two most common binding modes for Mg^{2+} (centre and left). The numbers over the snapshots indicate the number of oxygen atoms from −COO groups and phosphate groups of PS lipids involved in the binding. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

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Before showing the results, some technical considerations as well as recent studies have to be taken into account. The DNA molecule is a strongly charged polyelectrolyte; with a typical charge of one electron every 0.17 nm of its length [104]. This large negative charge plays a major role in many biological and technological processes. For example, adhesion and desorption of DNA onto solid surfaces can be controlled by tuning the charge of the surface [105]. In these experiments, large adhesion forces are obtained in the case of a positive electrode, and the adhesive force over DNA is completely suppressed by turning the charge of the electrode to negative. Interestingly, the addition of low concentrations of Mg^{2+} cations (which are well known as condensing agents of DNA [104]) does not induce adhesion of DNA onto a negatively charged surface.

The interaction of DNA with lipid membranes presents a much higher complexity, due to the self-assembled nature of lipid bilayers. Obviously, negatively charged DNA easily associates strongly with cationic lipids [4–9,18–21]. As we have discussed in a previous section, the case of negatively charged membranes containing anionic lipids is more interesting not only for its fundamental interest, but also due to its potential interest for biocompatible DNA delivery systems for gene therapy. Experimental evidences shown in Section 2 suggested that the interaction of a single DNA molecule with a membrane made only of anionic lipids (such as PS) is repulsive, even in the presence of divalent cations. As discussed in the previous section divalent cations are unable to invert the charge of a PS bilayer under reasonable concentrations (below the CCC) although they adsorb on PS membranes [53]. In other words, the PS membrane will remain with a net negative charge in the presence of cations. In any case, the binding of divalent cations onto PS membranes induces substantial conformational changes in the membrane and a complex (and highly inhomogeneous) charge distribution at the membrane surface, with positive patches coming from adsorbed cations and cationic amino groups also present in the anionic PS lipids. Amino groups are also known to interact strongly with DNA through ionic hydrogen bonds [105]. It is suggestive to think that these positive patches may act as putative binding sites for DNA in a negatively charged membrane.

This possibility has been confirmed by our recent MD simulations [106,107]. In these simulations, we obtain binding of a DNA molecule onto a pure PS monolayer by hydrogen bonding between the phosphate groups of DNA and the amino groups of PS (see Figs. 7 and 8). In addition, a small fragment of a DNA molecule is placed far from the PS monolayer (1.6 nm), in a solution containing low concentrations of CaCl$_2$. We added also enough Na$^+$ counterions to balance the charge of DNA and Ca^{2+} counterions to balance the charge of the PS membrane. The PS monolayer has a strong negative charge of 1.15 e/nm^2. However this charge density is almost neutralized by adsorption of Ca^{2+}, which has a strong affinity to the surface as discussed in the previous section. The charge of DNA is also renormalized by adsorption of cations. Hence, the strong electrostatic repulsion between these two negatively charged objects is greatly reduced due to both the adsorption of cations and the efficient screening by divalent salt. In the simulations, the DNA molecule initially diffuses far from the surface, as seen during the first 15 ns of simulation in Fig. 7. However, the low repulsive barrier between the DNA (with its adsorbed counterions) and the PS membrane (also with adsorbed Ca^{2+}) can be surpassed by simple diffusive crossing, as seen in the jump between 15 and 20 ns in Fig. 7. Once the DNA molecule is close to the surface, the DNA molecule approaches and patches onto the membrane containing positive charges (from lipid amino groups $-\mathrm{NH}^{+3}$ and adsorbed counterions) exposed to the solution. The atomistic detail given by the simulations reveals two different kinds of bonds between PS lipids and DNA molecules. In some of the bonds, Ca^{2+} cations participate as a bridge, linking a DNA phosphate with an acidic group of PS. However, the most frequent bond is a direct bond between the positively charged amino group of a PS lipid and a negatively charged phosphate of the DNA. In this case, also a Ca^{2+} is involved, but in this case indirectly. The PS lipids participating in these bonds have Ca^{2+} cations adsorbed at their negatively charged phosphate or carboxylic groups. Recall that, according to the results discussed in the previous section, PS lipids alter their conformations in the presence of divalent cations. In this new configuration, the positively charged amino groups (which interact strongly with DNA) become more exposed to the aqueous solution. In other words, adsorption of divalent cations by the membrane induces a structural change which makes possible the direct PS-DNA association.

4. Conclusions and future

The adsorption of DNA onto anionic lipids clearly plays an important role in the development of biocompatible DNA delivery systems for gene therapy. DNA–anionic lipids complexes constitute nowadays the most promising non-viric gene vectors. While the complexation of cationic lipids and DNA has been well studied, the use of less toxic anionic lipids for gene biomaterials has not hitherto been established. Moreover, addressing the interaction of DNA with a negatively charged membrane provides a unique platform for pure academic research, as fundamental knowledge of their structure–function relationships will afford a deeper understanding of their later performance as lipoplexes as genetic vehicles.

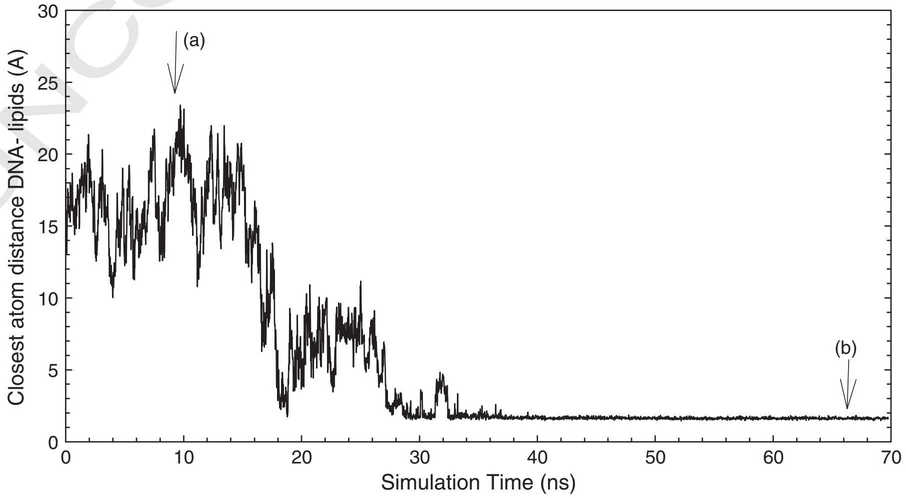

Fig. 7. Distance between a DNA molecule in electrolyte solution and a PS lipid monolayer as a function of time during MD simulations. The distance is calculated as the closest distance between any atom of DNA and lipid molecules. The arrows indicate the time corresponding to the snapshots shown in Fig. 6 (adapted from [107]).

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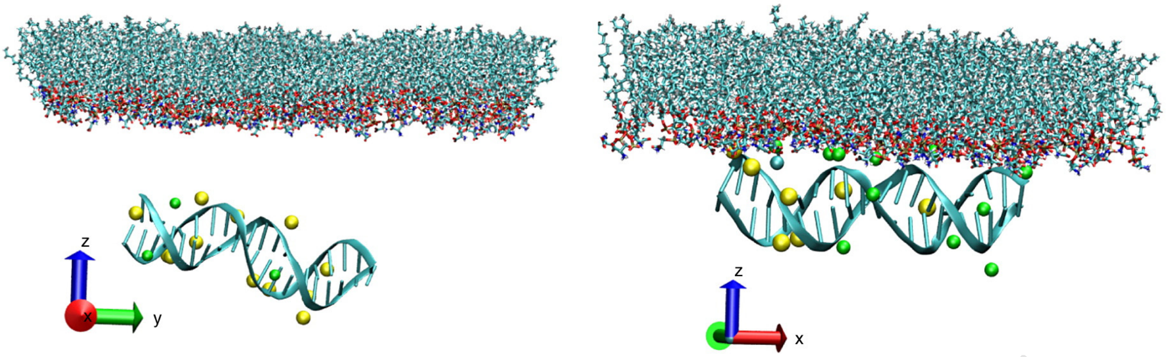

Fig. 8. Snapshots of MD simulations of DNA and PS lipid interaction at two different simulation times. Left and right images correspond to labels (a) and (b) indicated in Fig. 7. Lipid molecules are shown as bonds (colours correspond to standard crystallographic conventions). DNA strands are shown schematically and cations close to DNA are shown as spheres with Van der Waals radius (green corresponds to Ca^{2+} and yellow to Na^{+}). Case (b) is slightly rotated (as compared with (a)) in order to show more clearly the bonds between lipids and DNA. Figure made with VMD [108] (adapted from [107]).

This work combines a review of the state of art with the presentation of our new results in two sections: experimental and simulations. We first present the most important experimental studies concerning characterisation of anionic lipoplexes and their subsequent use in clinical transfection procedures, followed by new experimental results on the adsorption of DNA onto anionic lipid monolayers mediated by Ca^{2+}. Complementary to the experimental work we then review the theoretical framework of adsorption of DNA onto lipid membranes, which has limited to cationic and zwitterionic surfaces to date. This introduces new preliminary simulation work on DNA adsorption onto anionic lipid surfaces mediated by multivalent cations using two different computational methodologies. Although the simulations do not provide yet quantitative correlation with the experimental results, they do corroborate the findings. Namely, the displacement of the π-A isotherm obtained only in the presence of DNA and Ca^{2+} in the subphase can be interpreted as penetration of the DNA into the monolayer.

Combination of experiments and simulations sheds new light on the molecular mechanism underlying the binding of DNA with anionic lipids. However, further research is needed in order to understand the complexity involved in the bridging mechanism necessary to overcome the electrostatic repulsion. Even though this achievement is not a decisive result in final biomedical applications by itself, rational strategies can be developed to improve the transfer efficiency of lipoplexes as delivery systems *in vivo*. Accordingly, colloid scientists working in this field will be working increasingly in partnership with biologists and clinicians towards the rational design of low toxic nonviric gene vectors.

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