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Self-assembly of cationic liposomes–DNA complexes: a structural and thermodynamic study by EDXD

G. Caracciolo [a], R. Caminiti [b], D. Pozzi [c], M. Friello [a], F. Boffi [a], A. Congiu Castellano [a,*]

[a] *Dipartimento di Fisica and INFM, Università 'La Sapienza', Piazzale Aldo Moro 2, IT-00185 Rome, Italy*
[b] *Dipartimento di Chimica and INFM, Università 'La Sapienza', Piazzale Aldo Moro 2, IT-00185 Rome, Italy*
[c] *INFM, Unità di Roma 1, Università 'La Sapienza', Piazzale Aldo Moro 2, IT-00185 Rome, Italy*

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Abstract

We report a comprehensive study of the lamellar dioleoyl trimethylammonium propane and DNA complexes (DOTAP–DNA) as a function of the lipid/DNA weight ratio ρ in order to determine their structure and thermodynamic stability using an energy dispersive X-ray diffraction technique (EDXD). These self-assemblies consist of a periodic multilayer structure with DNA chains adsorbed between cationic membranes. Furthermore, above and below the isoelectric point, the DNA packing density significantly varies as a function of ρ. Our results confirm that EDXD is a powerful technique to characterize self-assembled ordered structures of oppositely charged macromolecules.

1. Introduction

The self-assembly of charged macromolecules into stable complexes plays an important role in the formation of many biological systems in which the interaction between the subunits is largely determined by electrostatic interactions. The spontaneous self-assembly of cationic liposomes complexed with DNA (CL–DNA), named lipoplexes, have recently attracted a great deal of attention due to their potential use as gene-delivery vectors [1]. It has been shown that CL–DNA complexes are comprised of liquid crystalline structures where a periodic one-dimensional (1D) lattice of parallel DNA strands is confined between two-dimensional (2D) lipid bilayers [2]. During complexation the cationic lipids neutralize the negative charge of DNA and allow the complex to approach the anionic surface of a cell.

However, the complex formation and its thermodynamic stability in solution cannot only be explained in terms of simple Coulomb attraction but its necessary to refer to the mechanism of counterion release. The counterion condensation (CC) models proposed by Manning [3,4] treat nucleic acids as uniform charged line with the phosphate groups partially neutralized by the

* Corresponding author. Fax: +39-6446-31-58.
E-mail address: a.congiu@caspur.it (A. Congiu Castellano).

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delocalized binding of counterions. The interaction of a positively charged ligand with nucleic acids causes a perturbation of the electrostatic potential of the charged surfaces with the result that some fraction of the Manning condensed counterions are released into the bulk solution with a remarkable entropy increase ($\approx 1k_BT$ per released counterion) [5]. CL–DNA complexes are often prepared using cationic lipids as condensing agents of the negatively charged DNA strands mixed with neutral helper lipids able to induce a specific topology with a larger colloidal stability by reducing the surface free energy [6]. Moreover, its well known that, when charged macromolecules are suspended at high densities, packing constraints lead to spontaneous self-assemblies to maximize entropy.

Otherwise, in the absence of added salt, the interactions are dominated by long-range repulsive forces and the spacings between the subunits of charged systems are set by a constraint. For example, the spacing between charged membranes, in the limit of zero salt concentration, is set by the finite volume available. In the case of lipoplexes the membrane charge density is the constraint that sets the equilibrium spacing between the DNA rods [7]. Recently, synchrotron X-ray diffraction (XRD) studies of the pure DOTAP–DNA complexes under free-salt conditions have been performed unraveling their supramolecular structure [8, and references included].

In this Letter we used the energy dispersive X-ray diffraction (EDXD) technique to focus the interaction between dioleoyl trimethylammonium propane and DNA (DOTAP–DNA) as a function of lipid/DNA (wt/wt) ratio ρ considering all the structural parameters. We have already applied EDXD to biological compounds and it has appeared to be of great help in studying the variation of ordered structures such as lipid bilayers [9] and proteins [10]. Even if synchrotron radiation is able to provide high-quality XRD scans because of its unique characteristics, our in-house XRD technique allows us to succeed in achieving essentially the same findings. Moreover, all the experiments can be any time performed in our laboratory in order to obtain useful informations about their repeatability without beam-time limits assigned by sinchrotron facilities.

Since we fail to consider the details of the lipid and DNA molecular structure, we expect CL–DNA complexes to represent a remarkable system model and our findings to apply to a wide class of self-assemblies formed nonspecifically between macromolecules because of the charge carried [11]. Its the case of the interactions between membranes and polymers, either adsorbed or tethered, which represent a fascinating area of research. In this context, quantitative studies about the nature of polyelectrolyte self-assemblies are highly desirable.

2. Experimental

2.1. Sample preparation

The CLs consisted of pure monovalent lipid DOTAP ($MW_{DOTAP} = 698.6$) having two 18-carbon (C_{18}) aliphatic chains per molecule. We used double-stranded calf thymus DNA ($MW(bp) = 649$). Both lipid and DNA were purchased from Sigma (St. Louis, MO).

Our samples for X-ray experiments were prepared dissolving 15 mg of the cationic lipid DOTAP in chlorofom. The chloroform was removed via rotary evaporation at 35 °C and the resulting thin lipid films were placed under vacuum overnight to ensure that all traces of solvent had been removed. The lipid was then resuspended in 1 ml deionized water at 45 °C until the film was hydrated, and then vortex mixed to afford an emulsion. We sonicated a deionized water solution of DNA 10 mg/ml for one minute inducing a DNA fragmentation whose length distribution, detected by gel electrophoresis, is between 500 and 2000 bp. By mixing an adequate amount of this solution with the suspension of cationic lipid 15 mg/ml, highly condensed self-assembled complexes at different ρ were obtained. The CL–DNA complexes are charge neutral when the number of DNA bases and DOTAP molecules are equal, i.e., when

$$\rho = \rho^{iso} = \frac{2 \times MW_{DOTAP}}{MW_{bp}} \approx 2.2.$$

2.2. X-ray diffraction

The X-ray experiments were carried out at the Physics Department by using an Energy Dispersive X-ray Diffractometer elsewhere described [12,13]. An incident polychromatic X-ray radiation is used and the diffracted beam is energy resolved by a solid-state detector located at a suitable scattering angle. The diffractometer operates in vertical θ/θ geometry and is equipped with an X-ray generator (W target), a collimating system, step motors, and a solid-state detector connected via an electronic chain to a multichannel analyzer. The X-ray source is a standard Seifert tube operating at 45 kV and 35 mA whose Bremsstrahlung radiation is used whereas the detecting system is composed of an EG&G liquid-nitrogen-cooled ultrapure Ge solid-state detector connected to a PC through ADCAM hardware. Both the X-ray tube and the detector can rotate around their common center where a cell is placed. After a preliminary set of measurements, four scattering angles, $\theta = 0.35°$, 0.55°, 0.7°, 4.5°, were selected to investigate the interesting range of the reciprocal space. The uncertainty associated to θ is $\Delta\theta = 0.01°$ and it directly affects the uncertainty Δq associated to q. Every spectrum has been collected at each fixed scattering angle θ for 10 000 s. Although the long acquisition time is an unfavorable aspect of this technique, no structural modifications and damage occur. Within the experimental error limits our XRD scans show the same features than synchrotron ones.

3. Results and discussion

We investigated the behavior of complexes in the limit of zero salt concentration as a function of ρ. All the XRD scans show two sharp peaks at $q = 0.103 \pm 0.008$ and 0.206 ± 0.02 Å^{-1} observed at $\theta = 0.35°$ and $\theta = 0.7°$, respectively. They correspond to the 0 0 1 and 0 0 2 Bragg reflections of an ordered multilamellar structure with a periodicity $d = 61.2 \pm 2$ Å. The uncertainty associated to d is simply calculated starting from q using the Bragg relation. Fig. 1a shows that d is almost constant as ρ varies from 0.5 to 20. Otherwise,

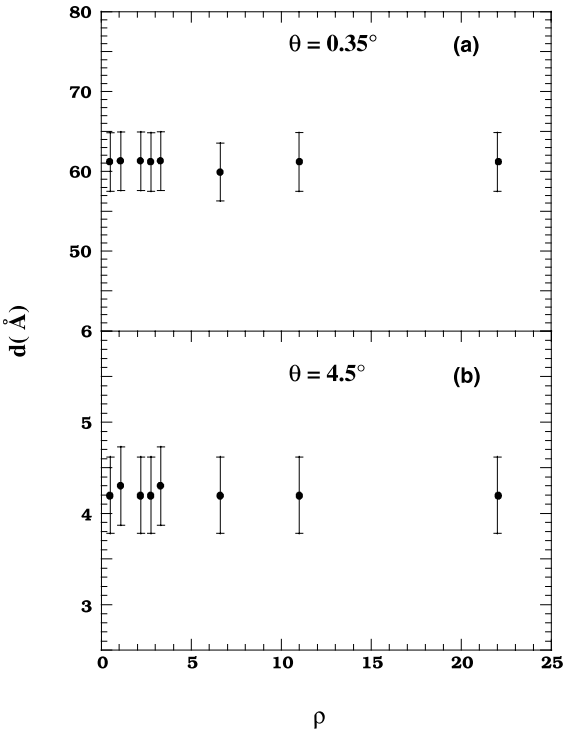

Fig. 1. (a) The spacing $d = \delta_m + \delta_W$ is nearly constant as a function of ρ as expected for a monolayer DNA intercalated in the water layers of lamellar stacks of lipid bilayers (for details see the text); (b) distance between the acyl chains of the lipid bilayer does not vary increasing with ρ.

fixing the scattering angle at $\theta = 4.5°$ we have covered a range of q from 1.0 to 3.5 Å^{-1} where the acyl chains present a strong diffraction peak due to their ordered structure. No structural modifications are observed and the distance between acyl chains does not change increasing ρ (Fig. 1b).This finding is directly interpretable in terms of DNA condensation which screens electrostatic interactions between lipid bilayers leading the system to condensed multilayers. DNA carries 20 phosphate groups per helical pitch of 34.1 Å and during complexation, the cationic lipid group heads neutralize the phosphate groups on the DNA backbone. Then, our XRD data are consistent with a topological rearrangement of cationic lipid and DNA into a multilayer structure with DNA double-strands intercalated between the charged lipid bilayers (L_α^c phase, Fig. 2). Since the lamellar repeat distance d does not vary as a function of ρ,

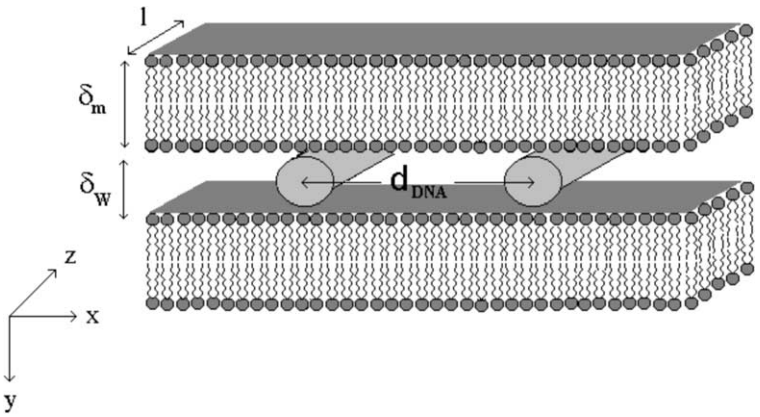

Fig. 2. Complex unit cell specified by the distance $d = \delta_m + \delta_W$, the DNA–DNA spacing d_{DNA} and the depth along the z direction l.

DNA remains tightly bound to lipid bilayers. Moreover, both experiments and calculations [14] show that the distance between apposed lipid surfaces δ_W does not vary as a function of ρ. It remains constant ($\delta_W \approx 25$ Å), as expected for a monolayer DNA including a thin hydration shell, so that the thickness of lipid bilayer $\delta_m = d - \delta_W = 36.2$ Å is simply calculated. The intermediate broader and weaker peak observed at $\theta = 0.55°$, which moves to lower q increasing ρ (Fig. 3), is clearly due to the interaxial spacing $d_{DNA} = 2\pi/q_{DNA}$. From the peak position the average interhelical spacing is obtained showing the existence of two different packing regimes: one where the complexes are positive ($\rho > \rho^{iso}$) and the other where the complexes are negative ($\rho < \rho^{iso}$). The former are strongly influenced by the interbilayer repulsions that set the finite amount of lipid that the complex can accommodate whereas the latter by interDNA repulsions. The complexes are one-phase in the region of the isoelectric point ($\rho \approx \rho^{iso}$), coexisting with DNA at lower ρ and with lipid at higher ρ so that d_{DNA} varies significantly from d^{iso}_{DNA} in both the regions (see Fig. 4).

Where all the DNA and lipids are incorporated into the complex d_{DNA} linearly increases with ρ and, at the isoelectric point, the free energy of the complex is minimal and the system is one-phasic and compressible. Beyond this point complexes continue absorbing excess cationic lipid and remain one-phase for $\rho > \rho^{iso}$ as long as lipids enjoy lower free energy into the complex with respect to that in the free bilayer. At some d_{DNA}, interbilayer repulsions forbid further accommodation of bilayer in the complex, the DNA–DNA spacing

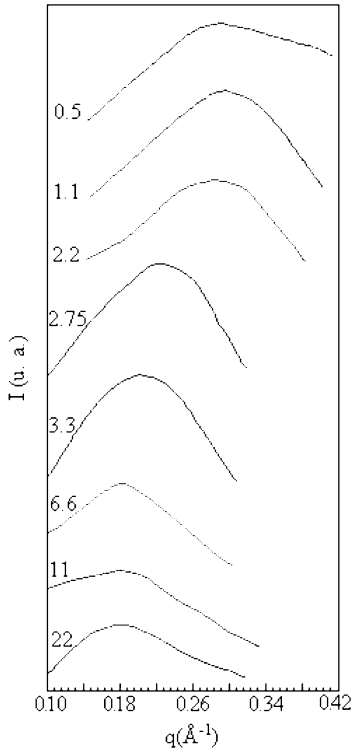

Fig. 3. EDXD scans collected at $\theta = 0.55°$. The DNA correlation peak moves to lower q increasing with ρ showing distinct DNA packing regimes: one where the complexes are positive ($\rho > \rho^{iso}$) and the other where the complexes are negative ($\rho < \rho^{iso}$).

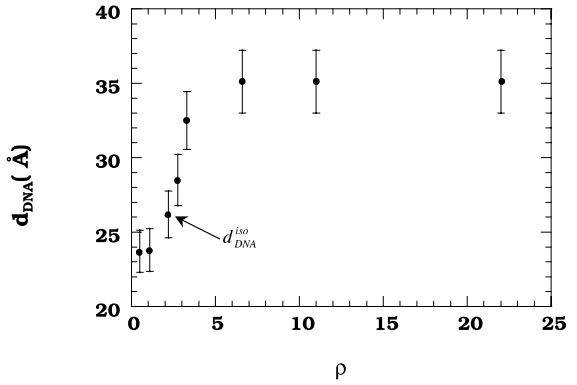

Fig. 4. Variation of the DNA-interaxial spacing d_{DNA} with ρ. The complex are one-phase across the isoelectric point coexisting with DNA at lower ρ and with lipid at higher ρ (for details see the text).

d^{+}_{DNA} does not vary anymore and the system becomes biphasic. Likewise one can determine the equilibrium interaxis distance in the complex d^{-}_{DNA} in the presence of excess free DNA. Since the free energy of a DNA strand in a complex is lower than in solution, additional DNA molecules enter the complex inducing the system to adjust d_{DNA}, in order to minimize its total free energy, as long as this overcrowding of DNA strands ($d^{-}_{\mathrm{DNA}} < d^{\mathrm{iso}}_{\mathrm{DNA}}$) is balanced by interDNA repulsions. In both these cases, the overcharging of the complex led to a new equilibrium condition starting from which the system is no more compressible. According to elsewhere reported models [15,16] we approximate the DNA strands as infinitely long parallel rods between planar lipid membranes, neglecting their possible curvature fluctuations in view of the fact that DNA persistence length ($\xi_{\mathrm{p}} \approx 500$ Å) is significantly larger than all of the other length scales in the complexes.

In order to explain the two above mentioned packing regimes one has to evaluate the energetics changes associated with DNA condensation in both the regions. The L^{c}_{α} phase is a periodic structure in the plane (x, y) perpendicular to the DNA axis (z). Because the complex is translationally invariant along the z axis, a unit cell is specified by the distance between apposed lipid surfaces δ_{W}, the thickness of lipid bilayers δ_{m}, and the DNA–DNA repeat distance d_{DNA}. The depth along the z direction, l, is the average spacing between negative charges on the DNA surface (along the B-DNA $l = 1.7$ Å) regarded as a cylindrical envelope of radius $R \approx 10$ Å with a continous and uniform charge distribution whose density is $\sigma^{-} = e/(2\pi R l)$. Each lipid molecule has one positive charge/head group of area $a \approx 70$ Å^2. Qualitatively [15] the complex unit cell may be regarded as a box with an uncompensated average positive charge on lipid surface for $\rho > \rho^{\mathrm{iso}}$ and negative charge on DNA walls for $\rho < \rho^{\mathrm{iso}}$. The curved DNA surfaces are approximated as planar surfaces of height δ_{W}, extending between the two planar bilayers. Considering an acqueous solution containing N^{+} cationic lipids and DNA strands of total length $l \times N^{-}$, N^{+}_{c} and N^{-}_{c} denote the cationic lipids and the DNA charges in the complex. Regarding the bilayer as a strip of width l and total length Γ and denoting as f_{c} the free energy of the unit cell of the complex, f_{d} the free energy of a naked DNA rod of length l and $\tilde{f}_{\mathrm{b}}$ the free energy of a bare bilayer of area $l \times d_{\mathrm{DNA}}$ per unit of length, its simple to write the total free energy of the system as a sum involving all the three possible phases

$$F_{\mathrm{c}} = N^{-}_{\mathrm{c}} f_{\mathrm{c}} + (N^{-} - N^{-}_{\mathrm{c}}) f_{\mathrm{d}} + (\Gamma - d_{\mathrm{DNA}} N^{-}_{\mathrm{c}}) \tilde{f}_{\mathrm{b}}, \tag{1}$$

whose nature is determined by minimizing this free energy with respect to all thermodynamic variables involved obtaining three different equilibrium conditions [15].

For $\rho > \rho^{\mathrm{iso}}$ the excess positive charge density on the bilayer surfaces is given by the dimensionless

$$\Phi^{+} = \frac{\Delta\sigma^{+}}{\sigma^{+}} = \left(1 - \frac{d^{\mathrm{iso}}_{\mathrm{DNA}}}{d^{+}_{\mathrm{DNA}}}\right),$$

whereas, for $\rho < \rho^{\mathrm{iso}}$, the excess charge density on the DNA walls is given by

$$\Phi^{-} = \frac{\Delta\sigma^{-}}{\sigma^{-}} = \frac{a}{2\delta_{\mathrm{W}} l}\left(1 - \frac{d^{-}_{\mathrm{DNA}}}{d^{\mathrm{iso}}_{\mathrm{DNA}}}\right).$$

In the above expressions

$$\sigma^{+} = \frac{e}{a} \quad \text{and} \quad \sigma^{-} = \frac{e}{2\delta_{\mathrm{W}} l}.$$

Treating all the box walls as planar surfaces, the closed-form expression for the electrostatic free

energy of a charged planar surface in the low salt limit [17] can be used

$$F = 2\left(\frac{2l}{a}\right)k_{\mathrm{B}}T\Phi\left[\ln\left(\frac{2l_{\mathrm{D}}}{l_{\mathrm{C}}}\right) - 1\right], \tag{2}$$

where l_{D} is the Debye length and l_{C} the Chapmann one. Furthermore, the electrostatic interaction between two equally charged surfaces separated by a distance δ_{W} ($\rho > \rho^{\mathrm{iso}}$) and d_{DNA} ($\rho < \rho^{\mathrm{iso}}$) has to be considered and the electrostatic free energy of the overcharging complexes, above and below the isoelectric point, assume the forms

$$f_{\mathrm{c}}^{+} = k_{\mathrm{B}}Tld_{\mathrm{DNA}}\left[\frac{4\Phi^{+}}{a}\left(\ln\frac{2l_{\mathrm{D}}}{l_{\mathrm{C}}} - 1\right) + \frac{\pi}{l_{\mathrm{B}}\delta_{\mathrm{W}}}\right], \tag{3}$$

$$f_{\mathrm{c}}^{-} = k_{\mathrm{B}}Tl\delta_{\mathrm{W}}\left[\frac{4\Phi^{-}}{a}\left(\ln\frac{2l_{\mathrm{D}}}{l_{\mathrm{C}}} - 1\right) + \frac{\pi}{l_{\mathrm{B}}d_{\mathrm{DNA}}}\right]. \tag{4}$$

The free energies of the bare bilayer and the naked DNA are simply given by the following expressions

$$f_{\mathrm{B}} = k_{\mathrm{B}}Tld_{\mathrm{DNA}}\left[\frac{4}{a}\left(\ln\frac{2l_{\mathrm{D}}}{l_{\mathrm{C}}} - 1\right)\right], \tag{5}$$

$$f_{\mathrm{d}} = k_{\mathrm{B}}Tl\delta_{\mathrm{W}}\left[\frac{2}{\delta_{\mathrm{W}}l}\left(\ln\frac{2l_{\mathrm{D}}}{l_{\mathrm{C}}} - 1\right)\right]. \tag{6}$$

Because $\Phi^{+} < 1$ so that $f_{\mathrm{c}}^{+} < f_{\mathrm{B}}$, the complex will absorb excess cationic lipid until stopped by the repulsive term in Eq. (3). Similarly, $f_{\mathrm{c}}^{-} < f_{\mathrm{d}}$ and the repulsive term sets again a limit on the amount of excess DNA that can enter the complex. Using the above expressions and the equilibrium conditions obtained deriving Eq. 1 with respect to d_{DNA}, one can directly obtain the set of equations

$$d_{\mathrm{DNA}}^{+} = d_{\mathrm{DNA}}^{\mathrm{iso}}\frac{1}{1 - \exp\left[-\left(1 + \frac{\pi a}{4l_{\mathrm{B}}\delta_{\mathrm{W}}}\right)\right]} \quad (\rho > \rho^{\mathrm{iso}}), \tag{7}$$

$$d_{\mathrm{DNA}}^{-} = d_{\mathrm{DNA}}^{\mathrm{iso}}\left[1 - \exp\left(-1 - \frac{2\pi\delta_{\mathrm{W}}l^{2}}{l_{\mathrm{B}}a}\right)\right] \quad (\rho < \rho^{\mathrm{iso}}). \tag{8}$$

From Eq. (7) it follows that $d_{\mathrm{DNA}}^{+}/d_{\mathrm{DNA}}^{\mathrm{iso}} \approx 1.37$ in the region $\rho > \rho^{\mathrm{iso}}$. Experimental values of $d_{\mathrm{DNA}}^{+} = 35.1$ Å and $d_{\mathrm{DNA}}^{\mathrm{iso}} = 26.2$ Å determine $(d_{\mathrm{DNA}}^{+}/d_{\mathrm{DNA}}^{\mathrm{iso}})_{\mathrm{exp}} \approx 1.34$ which is in good agreement with the predicted value. In the region $\rho < \rho^{\mathrm{iso}}$, $d_{\mathrm{DNA}}^{-}/d_{\mathrm{DNA}}^{\mathrm{iso}} \approx 0.85$ whereas the measured equilibrium distance is $d_{\mathrm{DNA}}^{-} = 23.7$ Å so that $(d_{\mathrm{DNA}}^{-}/d_{\mathrm{DNA}}^{\mathrm{iso}})_{\mathrm{exp}} \approx 0.90$.

In conclusion, our EDXD results have unambiguously confirmed that DNA is confined in 2D cationic membranes forming 1D lattice with a repeat distance d that does not depend on the lipid/DNA ratio ρ. We have found evidence that positively complexes have a lower DNA packing density ($d_{\mathrm{DNA}}^{+} > d_{\mathrm{DNA}}^{\mathrm{iso}}$) whereas the negative ones show a higher DNA packing density ($d_{\mathrm{DNA}}^{-} < d_{\mathrm{DNA}}^{\mathrm{iso}}$). Indeed, the electrostatic repulsions set a constraint on the excess lipid or DNA that the neutral complex can accommodate resulting as the driving forces for this spontaneous self-assembly. From a thermodynamic point of view, these experimental findings are interpretable in terms of the free energy changes between the initial and final states. More generally, these states could also correspond to the native and denaturated states in the proteins folding, to a single conformation at different ionic strengths for salt effects, to bound and unbound states of a ligand-receptor system. Furthermore, we have showed that EDXD can be successfully used to characterize complex structures.

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