Use of Cat-Anionic Vesicles as Molecular Vectors for Gene Transfer into Target Cells

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Abstract: We report on the possibilities to use cat-anionic vesicles as active vectors for transfection technologies. Cat-anionic aggregates are non-stoichiometric mixtures made of oppositely charged surfactants, or lipids. Depending on the relative amounts of two such components, bi- or multi-layered vesicles may be formed. The former ones adsorb bio-polymers on their outer surface, but bi-layers may also contain large amounts of lipophilic species in their interior. The transfection fate depends on the vesicle ability and efficiency in adsorption, which leads, eventually, to fusion with cell membranes. The intra-membrane uptake mechanisms differ significantly, and depend on whether the outer, inner, or bi-layer distribution of the species is considered. The first possibility involves a direct exchange of (supra) molecular entities between fluid surfaces in contact; the second and third, conversely, imply membrane fusion and subsequent transport of material within the cell matrix. A realistic combination of the above possibilities can be envisaged, and would ensure a long term action of the transferred (transfected) formulations. Examples taken from recent literature suggest that a realistic possibility is offered to high-yield molecular penetration: this becomes useful in gene transfer and molecular transfection technologies. Some technological aspects inherent to the above formulations are briefly outlined. The overall effect of penetration across the cell membrane of exogenous material in terms of biocompatibility is quite a formidable task to face with and shall be described in detail.

Keywords: Ionic surfactants, Ionic Lipids, Cat-Anionic mixtures, Vesicles, Lipo-plexes, Physico-chemical properties, Cytotoxicity, Transfection technologies, Bio-medical applications.

INTRODUCTION

Many efforts were made from the scientific community to develop effective bio-mimetic strategies for advanced medicine [1,2]. These require a systematic and interdisciplinary investigation, including some aspects of biophysics and physical chemistry, as well as of molecular and cell biology ones [3-6]. Each of the above fields of research is strictly interrelated with the others. In other words, multidisciplinary approaches help solving the fundamental problems and technical requirements inherent to the items of interest.

Targeting drugs, or complex formulations, to cultured cells or animal organs (though plants should not be disregarded with respect to this particular problem), requires a critical analysis of the advantages and drawbacks of each step associated to the fate of the mentioned material(s) therein. The rules dictated by biophysics and physical chemistry, thus, overlap with the biocompatibility requirements of complex living systems. For instance, the thermodynamic stability of the formulations conforms to the rules dictated by a significant availability and efficiency in the tissue organ. For pharmacological/clinical applications, in addition, accurate screenings must be carried out according to health-care requirements specific to any situation.

Different formulations were proposed in the past [7,8]. The most reliable ones must be endowed with the following properties:

a) Substantial thermodynamic and kinetic stability;

b) Well defined surface charge density;

c) Tunable dispersity in size;

d) Presence of regions acting as reservoirs of the cargo molecules to be delivered.

According to the above list (expressed in terms of hierarchical relevance), it is expected that micelles, water-soluble polymers, polymer-surfactant complexes, inorganic or organic nano-particles, co-acervates, vesicles, etc., fulfill some of the necessary requirements [9].

In this contribution, we report on a peculiar sub-class of supra-molecular aggregates, known as cat-anionic vesicles [10,11]. They are endowed with potential biocompatibility, and find application in gene targeting and drug delivery. Cat-anionic vesicles are formed by mixing non-stoichiometric amounts of ionic surfactants and/or lipids in water. We shall briefly illustrate how to prepare and characterize such lipido-

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mimetic systems (LMSs), promising platforms for efficient material transfer, biopolymer binding, and transfection. Vesicles and/or lipo-plexes (biopolymer-vesicle complexes, in this context), have sizes in the 100-500 nm size range [12,13], and are, therefore, suitable for applications at a cell-level.

The above entities are capable of significant matter exchange with cells, or tissues. A plausible uptake mechanism of such entities involves their adhesion onto cells and subsequent pynocytosis, or phagocytosis. When the active species is transferred to the cell, it will activate biochemical reactions involving metabolic pathways and/or homeostatic responses. Therefore, vesicles act as chaperons (N.B. The term chaperon, in this context, is considered in its usual meaning) for biopolymer or drug transfer across the cell membrane. To ensure a real biocompatibility, vesicles must be excreted and/or recycled at the end of the transfection process. Unfortunately, the scientific literature concerning this particular aspect is very scant. In any case, this is a relevant topic to be solved in future work and requires a detailed knowledge about cytotoxic features, possible chemical transformations generating noxious molecules within the cell matrix, cell membrane damage, and, finally, excretion/recycling pathways.

The properties of cat-anionic vesicular carriers make applications promising, since they ensure a more efficient transfection as compared to micelles, inorganic solids, polymeric or inorganic co-acervates, etc. [9]. In addition, vesicles are capable of transporting a variety of molecules such as hydrophobic species, polar drugs, and/or high molecular mass biopolymers. That makes a significant distinction with respect to the other vectors indicated above. Cat-anionic vesicles operate in a controlled way, and, very presumably, with no (or low) toxic effects. The most peculiar aspects of cat-anionic systems shall be exhaustively described in the following sections.

PHYSICAL CHEMISTRY OF VESICULAR SYSTEMS

The rationale suggesting the use of vesicular carriers for direct gene delivery is due to the combination of different factors, i.e.:

a) Significant adsorption onto vesicles of the biopolymer to be transferred [14];

b) Tunable physical state (gel, liquid or liquid crystalline) of the composites, similar to that of the cells;

c) Substantial and efficient binding of vesicles and/or lipo-plexes onto cells;

d) High biocompatibility towards cells and, in the long run, tissues [15];

e) Efficient uptake of hydrophobic drugs within the vesicles [16]

As mentioned above, cat-anionic vesicles, hereafter termed CAT-ANs, are formed by mixing in non-stoichiometric ratios cationic and anionic surfactants, or lipids, in water. The combination of electrostatic interactions between charged polar heads and of the hydrophobic ones among hydrocarbon tails jointly favors the formation of self-assembled entities. The occurrence of this phenomenon is related to the so-called “Critical Micellar Concentration” (CMC), which represents the minimum concentration at which surfactants in solution aggregate to form colloid entities with diverse morphologies [17]. [N.B. The term “Critical Vesicular Concentration”, (CVC), is still under intense debate]. Close to that limit, the diverse aggregate shapes essentially depend on the geometry of the individual surface-active species.

The relationship between the surfactant(s) molecular geometry and the shape of organized structures they form is quantified by the packing parameter, P [18]. The above value is representative of the shape that aggregates assume in the experimental conditions dictated by overall concentration, mole ratios, presence of co-solutes, temperature, pH, and/or ionic strength. Being the ratio between the hydrocarbon chain(s) volume, V, by the product of the mean area at the interface, A, multiplied by the main alkyl chain length, L, it is an a-dimensional quantity. The formation of vesicles is possible when P reaches a critical value, <1, with formation of closed bi-layers.

Under a strictly geometrical viewpoint, CAT-ANs are characterized by two radii of curvature, $R_{out}> 0$ and $R_{in}<0$, respectively, (with $|1/R_{in}| > 1/R_{out}$). Because of that, and given the small difference in modulus between two such radii, they are structurally similar to lamellar phases. It is possible, in fact, to observe concentric bi-layers with typical lamellar order and/or single bi-layered structures [19].

In the following section, we indicate how to prepare stable CAT-AN vesicles. We outline which procedures control their size and charge density, and the best conditions for an efficient biopolymer binding. In addition, the properties of a few selected systems,

recently proposed and utilized, are described. Details are reported on the preparation of cat-anionic vesicles based on lipids and/or surfactants. It will also be reported how to prepare niosomes, adducts with lipophilic drugs, and lipo-plexes, by adding vesicles with due amounts of biopolymers. The technical sides inherent to the aforementioned items can be found in specialized articles [20-22], which the reader is referred to.

Preparation Procedures and Fundamental Thermodynamic Aspects

Three preparative pathways are possible, in principle. They involve:

a) Mixing the solid surfactants in due amounts and diluting them with water, or saline media;

b) Dispersing a solid ionic surface active species in a proper solution of the other, and;

c) Mixing in due proportions oppositely charged surfactant (lipid) solutions having the same molality, respectively.

For a series of reasons, essentially imposed by the kinetics of dissolution and by solubility constraints, the latter possibility is to be preferred. It is not strictly required that the two solutions are at concentrations above the CMC, since the overall surfactant content required for vesicles to form is orders of magnitude lower than the respective critical micellar ones. This is because the interaction energy between the two components (quantified by a strongly negative interaction parameter, β) [23] gives much smaller critical concentrations than those predicted if ideal conditions of mixing occur. Therefore, CAT-ANs are extremely stable in terms of the Gibbs energy contributions to vesicle formation, i.e. $\Delta G_{f,Ves} << 0$.

The resulting dispersions have appearance ranging from bluish, typical of relatively small entities, to yellowish or lightly opalescent, and, finally, to milky, depending on vesicles' size. It is worth mentioning, on this regard, that it is formally possible to modify the macroscopic appearance of the dispersions changing the mole ratio, R, the temperature and/or the medium ionic strength. The rationale underlying the effect of temperature has a sound thermodynamic origin. Independently of the nature of the cat-anionic mixture, the two surfactants partition between the vesicular (pseudo)-phase and the bulk [24,25]. At constant temperature and composition, the chemical potentials of the single surfactants (indicated as μ_{AN} and μ_{CAT}, respectively) in each phase are the same. The partition between two such phases is different for the two surfactants; the more water-soluble one will preferentially transfer from the vesicle towards the bulk, and vice-versa. An increase in temperature favors the partition of the more soluble species toward the bulk (i.e. the entropy of transfer from vesicles to the solution, ΔS_{tr}, is positive). According to experiments, the above process is nearly irreversible [26]. This implies changes in vesicle composition and possible variations in size. The same considerations apply as to whether vesicles are multi- or single-layered.

The ionic strength reduces the solubility of the single surfactants in the bulk. It also modifies the vesicle' net charge, and its electrical double layer thickness, as well. The combination of the mentioned effects (i.e. R values, T, and ionic strength) may imply a regular and significant growth in size. It is also presumed that the presence of electrolytes increases vesicle rigidity, because of a reduced charge density.

By using one of the procedures mentioned above, or a combination thereof, size modulation is feasible. It must be kept in mind that diameters lower than 400 nm are to be preferred for practical purposes. In other words, vesicle size/volume must be substantially lower than the one of cells, for transfection to be effective. Moreover, adsorption or uptake of biopolymers onto vesicles may largely modify the size of the resulting lipo-plexes [27,28]. More details on the latter items shall be exhaustively discussed in a forthcoming section.

Drugs Uptake and Protein Adsorption

Given the very special nature of CAT-ANs as reservoirs and molecular vehicles, one can envisage that they may uptake exogenous materials in different ways and physical locations, namely;

a) On the outer surface;

b) In the bi-layer interior;

c) In the polar vesicles lumen.

Presumably, more such states can be concomitant. For physical consistency, we discuss them separately. The first is related to non-specific adsorption onto charged surfaces and is related, very presumably, to electrostatic forces. The second is due to the establishment of hydrophobic interactions between the

drug and the palisade vesicle layer; it is mostly intended for non-polar, or partly polar, drugs. The third kind of interaction between vesicles and cargo-molecules requires special procedures to be settled in; for instance, through formation of lipo-plexes and re-dispersion of the latter into an oppositely charged surfactant solution.

Polymer adsorption on the outer vesicle surface requires the onset of significant electrostatic interactions among the vesicle surface and the species to be adsorbed. Current procedures mostly apply to polyelectrolytes, proteins [29], and nucleic acids [30]. Depending on the vesicle net charge, electrostatic interactions occur between the vesicle surface and that of the adsorbing species. As expected, pH and ionic strength are a key-point in such cases. For instance, the binding of albumin onto oppositely charged vesicles is strongly pH-dependent [14]. The latter variable not only affects the electrostatic contributions to binding, but also the conformational state of adsorbed protein. Charge selectivity also holds in case of DNA or RNA binding onto oppositely charged vesicles, and can be properly controlled.

As to the uptake of hydrophobic drugs into the vesicle bi-layer, it is conceivable that they are somehow anchored onto the vesicle surface and partly embedded in the related bi-layers. Typical examples of the categories fulfilling such behavior are steroidal drugs [31]. To distinguish them from lipo-plexes, we refer to such category as niosomes. They are prepared as follows. Due amounts of the two surfactants, possibly added with cholesterol, are dissolved in chloroform, evaporated and vacuum dried. Thereafter, they are hydrated in presence of water, or dispersions of the drug to be encapsulated, at adequate temperatures. They are allowed to equilibrate until a complete partition of the drug is attained. Dialysis follows, to remove the drug in in the bulk. More details on the preparation procedures are reported elsewhere [16,32].

Concerning, finally, the preparation of vesicles containing biopolymers in the inner core, there are still substantial technical problems under debate. Internalization may require the formation of pores in the vesicle bi-layer; this hypothesis is not energetically favored, until special conditions are met. One possible procedure implies thermal fusion (eventually sonication-assisted) of vesicular dispersions to which a biopolymer is added [33]. Yields are moderate, usually a few percent, but do not require dialysis. In the case of proteins, conversely, dialysis is necessary to reduce their presence in the bulk and to rule out different uptake pathways in the cells. Another possibility relies on the formation of lipo-plexes and re-suspension in a solution of the second surface-active species. Conditions for this way to be fruitful is that the concentration of lipo-plexes is moderate, and no thermal treatments are performed. The latter, in fact, may induce the systems to undergo thermal gelation [34].

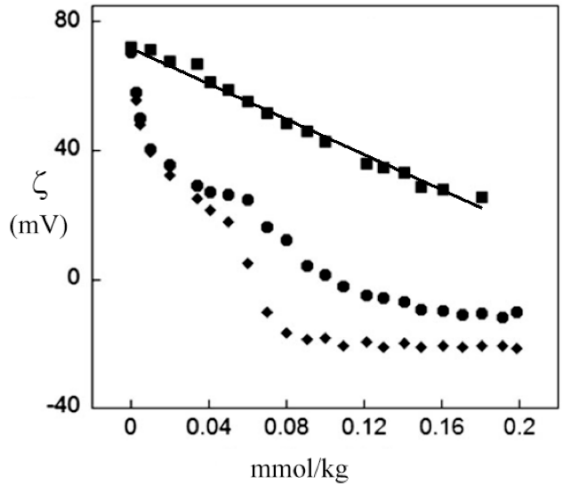

Figure 1: Albumin adsorption onto negatively charged vesicles (the concentration is 4.05 mmol kg^{-1} and the R ratio 3.80/1.0 [DDAB/SDS]) as a function of protein concentration, in mmol kg^{-1}, at 25.0 °C. Data refer to pH 4.0 (full squares), 6.8 (full circles) and 10.0, respectively. Comparing the latter two cases indicates a shift in the saturation threshold, i.e. the inflection points in the curves. Data at pH 4.0, conversely, are a linear combination of the electrophoretic contributions due to vesicles and free protein. In fact, albumin has positive charges in excess, in such conditions.

Electrostatic Interactions

As mentioned above, biopolymer adsorption onto CAT-ANs is essentially ruled by electrostatics, and, in some cases, hydrogen bond interactions. The former hypothesis is strongly supported by ad hoc experiments unequivocally showing that, in case of proteins, the vesicle surface saturation threshold and the amount of adsorbed biopolymers are regulated by pH [35]. That is, the amount of polyelectrolyte required for an effective, complete, adsorption is dependent on its charge and on the number of sites onto which it interacts, Figure **1**. It also is evident from ad hoc CD experiments that conformational changes are a combined result of the medium acidity and adsorption onto oppositely charged entities [35].

In addition, the ζ-potential and the surface charge density, σ, as well, change regularly upon protein

addition, until a sort of saturation is attained; above it both values do not change any more. The saturation threshold corresponds to the electro-phoretic mobility limit (i.e. to the ζ-potential) pertinent to the free biopolymer in presence of titrated vesicles.

Data in the whole concentration region that is under scrutiny can be elaborated in terms of an adsorption isotherm, a Langmuir-like one, for instance. That procedure gives the opportunity to evaluate the surface saturation limit, and the formation of a mono-layer onto vesicles. The surface saturation threshold, θ, is always less than unity [36], as indicated in Figure **2**.

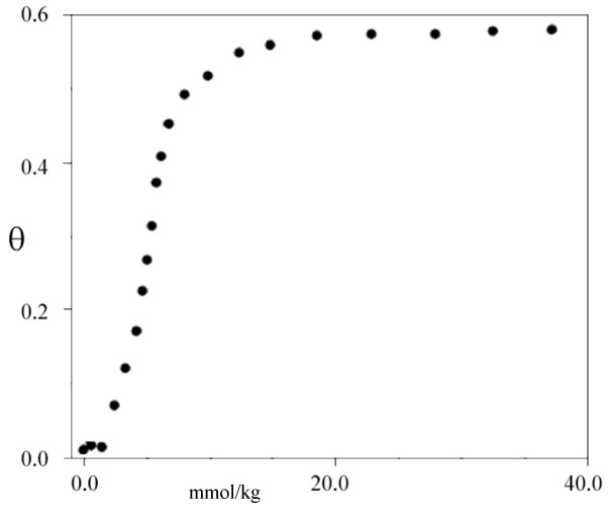

Figure 2: Surface coverage, θ, as a function of added poly-L-Lysine hydrobromide, (in mmol kg^{-1}), to the DODAB/SDS system. The vesicle concentration is 15.0 mmol kg^{-1} and the [SDS/DODAB] mole ratio 3.32/1.0. Data were elaborated from electro-phoretic mobility measurements.

ζ-potential and surface charge density, σ, scale with the mole ratio between surfactants, R, Figure **3**. When the latter → 1.0, ζ and σ tend to zero, with occurrence of a macroscopic phase separation. The same holds when bio-polymers interact with vesicles. The charge neutralization processes occurring upon addition of polyelectrolyte, or of the species in defect, find analogy to classical colloid titration. Significant similarities, for instance, can be found with the titration of silica, or titania, with oppositely charged polymers [37,38].

We do not consider what happens when |ζ| < 25-30 mV, Figure **3**. That threshold implies the onset of a kinetic instability, related to fact that the ratio of electrostatic to thermal energy is close to unity, or lower. At that limit, the vesicular dispersions or lipo-plexes are destabilized and a macroscopic phase separation occurs, with sedimentation or creaming.

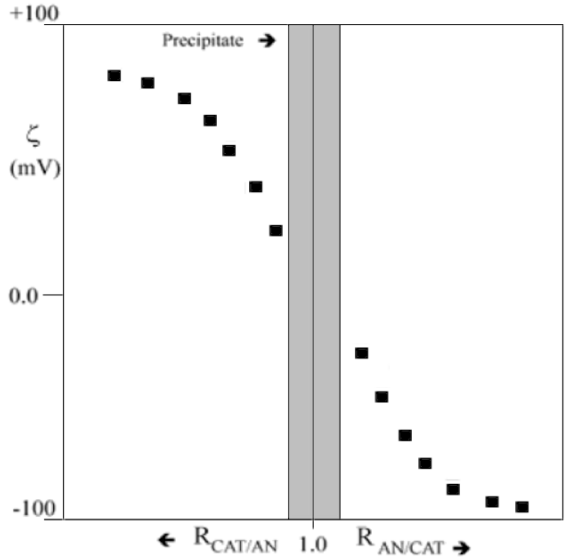

Figure 3: Dependence of zeta potential, ζ (mV), on the R ratio in 15.0 mmol kg^{-1} [AOT/DODAB] vesicles, at 25.0 °C. Data on the left refer to positively charged vesicles, and the reverse holds in the right hand side part of the figure. Note the presence of a precipitate area, in gray color, when R is close to unity.

Free surfactant ions, counter-ions, salts released from vesicles, and added electrolyte jointly contribute to the medium ionic strength and to the double layer thickness. They exert a significant effect on ζ-potential, due to changes in the medium and surface charge neutralization. For instance, addition of 25.0 mmol kg^{-1} NaBr to the DODAB/AOT system, when R = 8 and [CAT+AN] is 15.0 mmol kg^{-1}, results in changes of ≈ 80.0 mV, Figure **4** [39]. Above that electrolyte content

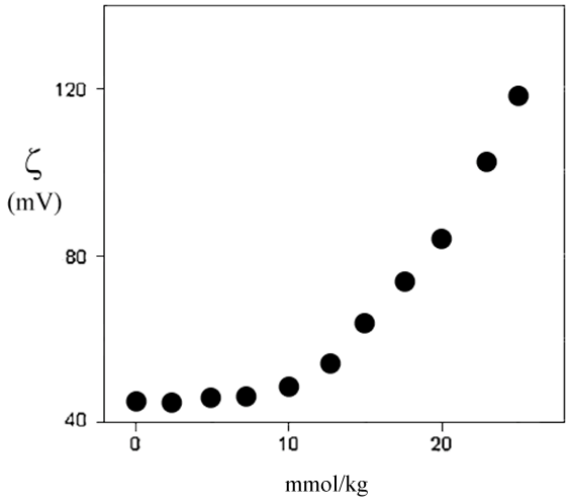

Figure 4: Dependence of zeta potential, ζ (mV), on the molality of added NaBr in 12.5 mmol kg^{-1} [DODAB/SDS] vesicles, at 25.0 °C. The mole ration between the surfactant components is 7.30/1.0. Note the nonlinear dependence on ionic strength. Above 25.0 mmol kg^{-1} NaBr vesicles size diverges and phase separation occurs.

the surface charge density reduces and lamellar dispersions are formed. In the latter region it is not possible to detect reliable ζ-potential values.

Both the charge distributions and electro-kinetic effects are associated to ion motions toward, from, and around colloids. The surface charge density, σ, is the time-averaged value of all such contributions. It is obtained from ζ-potentials according to

$$\sigma = \left[\frac{\varepsilon \zeta}{4\pi\delta} \right] \quad (1)$$

where δ is the double layer thickness. To give more physical consistency, Eq. (1) is rewritten as

$$\sigma\delta = \left[\frac{\in \zeta}{4\pi} \right] \quad (1')$$

to put in evidence that ζ is proportional to an electric moment per unit area.

The distance between the surface of charges on the vesicles and the slippery plane is equivalent to the double layer thickness, δ, and depends on ionic strength, I, including that due to the biopolymer. In the following we shall demonstrate that δ is also related to the dielectric relaxation time. The dependence of δ on composition can be inferred by deriving Eq. (1') with respect to the amount of added electrolyte, polyelectrolyte, or as a function of R. In all such cases, when $\zeta \rightarrow 0$, the same holds for σδ. At that point, σ approaches zero and δ diverges. That is, no ion-vesicle (lipo-plexes) electrostatic interactions occur if the latter are uncharged.

In case of polyelectrolyte binding, the amount of released ions depends on the extent of titration. We do not have realistic estimates on the absolute values, since ionic mobility in the bulk depends on the presence of all ions. Perhaps, it is possible to quantify how binding depends on added electrolyte, biopolymer or R. On this purpose, surface binding models based on ζ-potential measurements were analyzed by a Langmuir-like approximation, and properly adapted to ion adsorption onto oppositely charged surfaces. The surface charge density, σ, can be expressed as [40].

$$\sigma = \left[1 - \left(\frac{z\vartheta_s}{C_{el}} \right) \right] \cdot \left(\frac{e}{A^0} \right) \quad (2)$$

where $z \cdot e$ is ion charge, C_{el} the concentration of added electrolyte, A^0 the area per binding site (usually 0.6±0.1 nm^2), θ_s the surface coverage, and ($e/A^0 = \sigma^0$) a surface charge per binding site. Rearrangement gives

$$\theta_s = \left(\frac{C_{el}}{z} \right) \cdot \left[1 - \left(\frac{\sigma}{\sigma^0} \right) \right] \quad (2')$$

linking changes in σ and θ_s to the ionic strength. Similar relations hold in case σ is related to R. In the systems considered here the increase in θ_s is over 20% for an ionic strength < 20-25 mmol kg^{-1}. $d\theta_s$ slightly depends on ionic strength and levels off at the above electrolyte content.

Changes in ion binding are not necessarily a linear function of salt, or polyelectrolyte, content. This may be the reason for the non-linearity effects observed in ζ-potential trends reported in Figure **4**. Presumably, ion condensation is large if ($\partial\sigma/\partial$salt) is large (for low amounts of titrant), and negligible at high content. In words, surface charge saturation progressively takes place. A significant charge neutralization of vesicles takes place at the phase boundaries. This occurs when R ratios are close to unity, but also when ion or polyelectrolyte concentrations are such to neutralize vesicles.

The situation is very much the same when proteins or nucleic acids are added to vesicular dispersions. Experiments along the latter line were performed in case of double strand DNA [41] ζ-potential data are also supported by dielectric relaxation ones. Studies reported so far deal with uncharged mono-disperse hard spheres in supporting electrolyte. In cases like such dielectric relaxation gives information on the double layer thickness [42]. The double layer contribution to conductivity is related to the polarizability amplitude, *P**, and the dielectric constant increment reflects the polarization dynamics. On increasing the frequency, the dielectric increment scales with the double layer thickness, when the reverse behavior holds for the conductivity increment. The two contributions dominate at low and high frequencies, respectively. The high frequency behavior of Δε does not automatically match the double layer dynamics. The situation can be largely different when charge-modulated poly-disperse entities are dealt with. It is un-proper to assume extra constraints or adjustable fitting parameters to available theories, and we rely on a simple approach.

At first, the frequency-dependent permittivity is calculated in equilibrium conditions. An AC electric field is then applied and the corresponding values

recalculated. The relations are expressed as $A^{\pm} = A^{\pm\circ} + dA^{\pm} + (dA^{\pm})^2$ …, where $A^{\pm}$ is the relevant quantity and higher terms are immaterial: the reasons for that shall be described below. AC fields modify the electrostatic potentials (ϕ), the number of ions (n), the osmotic pressure (Π), the charge density (ρ), ion velocity (v), matter fluxes (J), and chemical or electro-chemical, potentials (μi, or μi*). The differentials are linearized and high order terms neglected: thus, a linear perturbation regime holds ($dA^{\pm} << A^{\pm\circ}$).

Theoretical approaches reported so far allow getting information on the properties to be considered. Some deal with intrinsic colloids, other with association ones. In micellar colloids, for instance, models linking dielectric relaxation to composition were proposed [43]. However, micelle sizes are orders of magnitude smaller than vesicles and their behavior; therefore, they do not conform to Smoluchowski's approximation (i.e. δ> D_H/2). Thus, new routes must be considered. Among those reported to date, Shilov's one is the most reliable [44]. Theories based on such approach [45,46], allow to derive the Poisson-Boltzmann equations for n, ρ, and ϕ; the above quantities are related to σ and/or polarization.

In these theories, the self-diffusion coefficients of ions, D^+, D^-, were accounted for. Even though the approximation is not realistic, D values are supposed to be responsible for radial and tangential ion motions around vesicles. D^+ and D^- are sensitive to the type of ions in the medium. The contribution due to free surfactant ions or polyelectrolytes is of minor relevance in presence of neutral electrolytes. We performed some calculations and found that the error induced by the above approximation is < 5%.

The dependence of dielectric increment on the volume fraction of the disperse phase, φ, can be written as

$$\left(\frac{\in -\varepsilon^{\circ}}{3\varphi\varepsilon_{\infty}}\right) = Re(P^*) + \left(\frac{\chi}{\omega \in_{\infty}}\right).[Im(P^*)] \quad (3)$$

where χ is the conductivity term, φ the volume fraction of the disperse phase, and *P** the polarization one. Their values depend on φ (more correctly on particle sizes). Eq. (3) can be split in two or three terms, depending on whether Maxwell-Wagner contributions are accounted for. Accordingly, it can be expressed in implicit form as

$$\left(\frac{\varepsilon - \varepsilon^{\circ}}{3\varphi\varepsilon_{\infty}}\right) = Re(P^*) + Im(P^*)_{\chi} + Im(P^*)_{MW} \quad (4)$$

where the first term refers to the dielectric increment, the second to conductivity, and P^*_{MW} to an eventual Maxwell-Wagner contribution. *P** values depend on the double layer thickness. In case a single population of particles exists, *P** can be expressed as

$$\left(\frac{P^*}{P^{\circ}}\right) = \left[k \cdot \left(\frac{D_H}{2}\right)\right]^3 \quad (5)$$

where κ is the inverse screening length and P° a reference value.

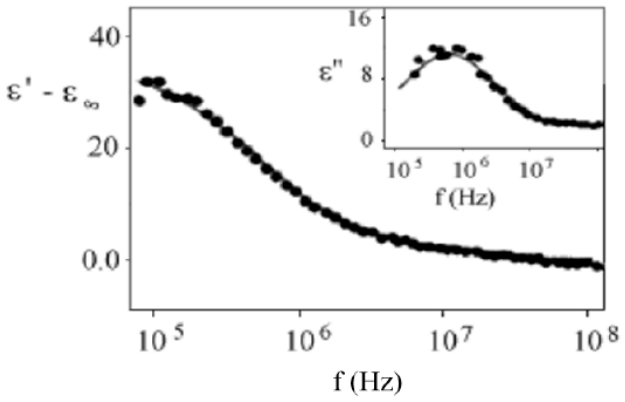

Figure 5: The real, full figure, and imaginary, inset, dielectric relaxation spectra of a 6.00 mmol kg^{-1} SDS-CTAB vesicular dispersion of R ratio equal to 3.10/1.0, at 25.0 °C.

According to Eq. (5), d*P** is proportional to $d\kappa^3$; that is the polarization amplitude is related to the number and length of dipoles facing outward the vesicle surface (i.e. to the sum of electric moments facing outward vesicles). The radial relaxation mode depends on I (on δ, therefore), when the tangential one is much slower, being related to the motion of ions in the slippery plane around vesicles. Estimates based on f* values indicate that former hypothesis is realistic. According to what stated in the experimental section, no explicit Maxwell-Wagner terms were observed in the available frequency window. This allows considering only canonical contributions to the permittivity, provided due functional relations are present in Eq. (3).

In terms of Eq. (5) *P** depends on poly-dispersity and is related to the effective <D_H> values, when *κ* is a constant (if the ionic strength is kept fixed). For vesicles having sizes between 200 and 500 nm in variable amounts, for instance, changes in (*P*/P°*) are over one order of magnitude, depending on the relative weight of the two populations. The above behavior is reflected by

significant changes in dielectric properties, as in Figure **5**.

Vesicular Systems in Biology: Biocompatibility Characterization and Ribonucleic Acid Trasfection and Potential Applications

This section will focus on the evaluation of the biological activity of lipo-plexes, especially with respect to possible consequences of particle entry, cell survival after exposure to these particles, and cargo molecule delivery upon transfection. We will examine cell viability and vesicle-induced cytotoxicity after uptake of exogenous bio-macromolecules; in our case messenger RNA. Also, possible membrane damage occurring during and/or after trans-membrane penetration of the vesicle (lipo-plexes) will be examined.

The results discussed in this section summarize in a critical fashion recent results from our laboratory. We hope that this work will be useful to researchers engaged in this field, but also to readers more generally interested to vesicle biocompatibility and the intracellular delivery of exogenous molecules.

The lipo-plexes formed upon interaction of vesicles with bio-macromolecules, such as DNA, RNA, or proteins, represent a potential tool to deliver genetic material across the plasma membrane, thus modifying the genetic features and metabolic potentialities of the recipient cell. Complexes between positively-charged vesicles and bio-macromolecules occur when the latter ones expose a net negative charge, as, for instance, DNA and RNA. These complexes are delivered within the cell. This latter, after exposure to the action of exogenous carriers (vesicles or lipo-plexes), as well as of their isolated components, may suffer cytotoxic effects, depending on dose and time of treatment. However, the possibility that the cargo molecule may have per se a noxious effect should be taken into account, as well. Also, the nature of the cell may play a role in the sensitivity to the treatment. Concerning this latter aspect, literature is somewhat deficient, but recent work from our laboratory showed that tumor cells exhibit a higher sensitivity to treatment with SDS-CTAB vesicles as compared to normal mouse fibroblasts [15,48-51]. In proper experimental conditions an efficient vesicle-mediated transfection may also lead to the expression of exogenous genetic material, as shown by pilot-studies suggesting a possible use of cat-anionic vesicles in anticancer therapy [52-54].

In the light of just discussed, we review here the cytotoxic action of individual surfactants and their cognate vesicles on cultured cells. Also, we discuss data on the transfection of an exogenous RNA mediated by vesicles: the transfection results in a high level of cytoplasmic production of the reporter-protein. The nucleic acid translated into protein has, in addition, the correct configuration since it immuno-reacts with its specific antibody, thus suggesting that these vesicle-cargo complexes are potentially usable in biotechnology and gene therapy. However, after administration of lipo-plexes to cultured cells, a cytotoxic effect with consequent activation of the controlled cell death pathway is observed. Therefore, we envisaged that a possible damage to the cell membrane may occur during the process of penetration as previously reported also by our laboratory [15]. With respect to this, we show that liposomes, supra-molecular aggregates comparable under the chemical and dimensional point of view to vesicles, are able to traverse the cell plasma membrane without causing relevant consequences on its structure and function [55,56]. In the following discussion we give a reasonable interpretation for this apparent discrepancy.

In any case, a study of the potential use of vesicles and related supra-molecular aggregates in nano-biotechnology is impossible without a previous evaluation of their impact upon living cells. The first effect that should be examined is the cytotoxic action exerted by vesicular suspensions and lipo-plexes. In any case, cytotoxicity is a unique feature for each vesicle type and depends on a number of diverse parameters such as chemical composition, nature of the surfactants, vesicle size, and, also, on the nature of the cargo molecule, just to mention but a few. The cellular/molecular phenomena underlying the toxic effects and subsequent cell death should also be elucidated. Therefore, we shall examine the damage at DNA level. This is a molecular event occurring in the advanced stages of cell death by apoptosis, as briefly discussed below. In particular, the molecular aspects of this phenomenon will be illustrated. A cell dies according principally to two different modes: apoptosis and necrosis, even though other phenomena as necroptosis and autophagy have also been considered. All the facets of these cell death modes have been extensively reviewed; therefore we address the readers particularly interested in these fascinating topics to already published “classical” and recent works [57-62].

Surfactants cytotoxicity is exerted, as mentioned above, at differential extent, depending upon cell line,

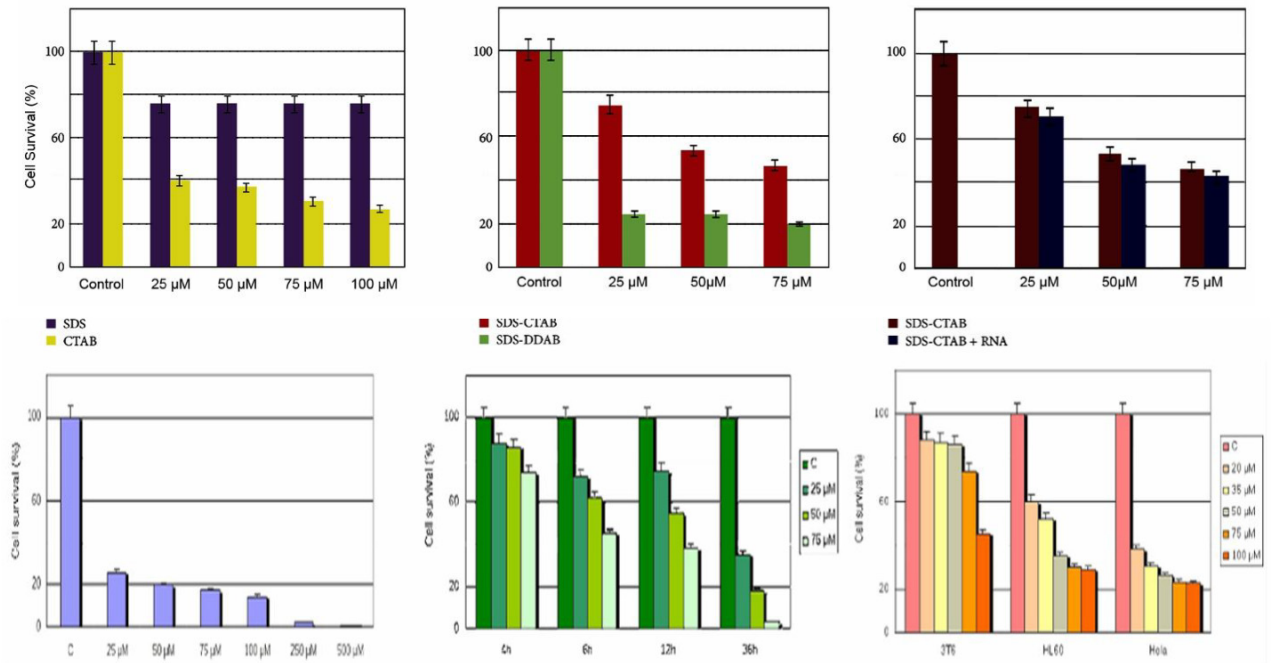

Figure 6: Effect of the separate surfactants and dose, time, and differential sensitivity to SDS-CTAB vesicles vesicles on the viability of cultured cells HEK-293 cells. Upper Row: Left panel: Toxic effects of the individual vesicle components SDS (purple bars) and CTAB(yellow bars). Center panel: Compared cytotoxicity of cat-anionic vesicles formed with SDS-CTAB (red bars) and SDS-DDAB (green bars). Right pane:) Cytotoxicity of vesicle/RNA lipoplexes, i.e vesicles loaded with RNA as cargo molecule. In all cases a normal strain of Human Embryo Kidney (HEK293) cells was used. The rational for this choice was dictated by the higher susceptibility to transfection shown by this cell line, as commonly accepted by literature data. Lower Row: Left panel: Cyto-toxicity of vesicles after 24 hours of treatment at increasing vesicle doses. SDS-CTAB vesicles show a pronounced cytotoxicity even at a concentration as low as 25 µM. Center Panel. Vesicles ctyo-toxicity as a function of concentration and time of treatment. The murine fibroblast 3T6 line was grown in the presence of vesicles at the indicated concentration and time. Right Panel) Cyto-toxicity of cat-anionic vesicle on different cell lines. Cells were treated with vesicles for 4 hours. After this treatment the cytotoxic effect of vesicles is minimal (see results of the previous figure and the discussion in the main text, section: Short-term reversible membrane damage and Cell death: making ends meet). However, at longer treatment times higher cell death takes place on tumor cells (HeLa and HL60) as compared to normal ones (3T6). In all cases, cell viability was assessed by the colorimetric Mossman assay [70]. The error bars indicate the Standard Error of the Mean.

dosage and time of treatment. Figure **6** summarizes the effects of these different parameters. A few conclusions can be drawn, i.e. CTAB is more toxic that SDS; the mortality rate is directly proportional to the concentration of both surfactants. Finally, the effect of cat-anionic vesicles is dose and time-dependent. Short treatment times do not affect the cell survival in a relevant way. It is also worth noting that human tumour cells lines are in general more sensitive with respect to normal ones [15,52]. This is reasonably due the different structure of the tumour cell plasma-membrane as compared to the normal one [63,66].

Permeability may play a crucial role since the plasma membrane fluidity in tumour cells is apparently higher than in normal ones [15,62-66]. Tumour cells do not respond significantly to treatment at low vesicle concentration; a plausible way to interpret this result is that these cells are not homogeneous, but include an intrinsically more resistant sub-population. Unpublished results from our laboratory show that DDAB is per se significantly more toxic than CTAB. It is also evident that SDS-DDAB vesicles are by far more toxic than SDS-CTAB ones. The higher toxicity can be realistically due to the intrinsic noxious action of DDAB. With respect to this point, it should be pointed out that that a fraction of non-vesicle associated amount of the individual surfactants is present free in solution (see also the published phase diagram) [67]. The latter is responsible for cito-toxicity effects, in proportion to its effective concentration in the bulk. It is it also possible that the association of the two chemical species may play a synergistic role as far as cytotoxicity is concerning. In the light of the results reported here, it is evident that SDS-CTAB vesicles are good potential candidates for the delivery of cargo molecules within an industrial and/or biomedical field of application. One conclusive consideration: the cytotoxicity of SDA-CTAB/RNA lipo-plexes results in a slight increase in cell mortality: this may be ascribed to the commonly accepted notion that free RNA may have a toxic action for the target cells. One further point to examine is

whether cytotoxicity is directly involved in a possible DNA damage. Specific TUNEL assays [15,67,68], conducted in our laboratory, show that DNA undergoes a severe fragmentation in vesicle-treated cells, as visualized by fluorescent labelling both at single strand and double helix level, Figure **7**. A heavy DNA damage is not compatible with cell survival, as commonly accepted by the scientific community [68,69]. However, the combined data of toxicity tests, measured by MTT [15,70], and TUNEL assays [15,66,68,71-74], do not rule out the possibility that also a defective cell proliferation is observed in parallel to actual cell death.

Cell Death after Exposure to Vesicles: Plasma Membrane Alteration and Activation of the Apoptotic Pathway

The MTT [70], and the TUNEL [75] assays are suggestive of extensive cell death; therefore, we also investigated the level of membrane lipo-peroxidation which is another specific marker of cell death occurring by apoptosis. Increased peroxidation levels of membrane lipids are, as a matter of fact, strongly suggestive of the onset of this phenomenon. That chemical reaction occurs in response to oxidative stresses and consequent damage of the plasma-membrane, and takes place only in the cells exposed to the stress when Malonal Di-hAldehyde (MDA) is produced. The quantitative measurement of the intracellular concentration of MDA, which is not present in healthy cells but derives from the peroxidation of poly-unsaturated fatty acids present in the plasma membrane, provides directly the degree of membrane lipoperoxidation and its influence on membrane fluidity consequent to oxidative stress [62-66]. More specifically, this molecule reacts with the free amino-groups of proteins embedded in phospholipid bilayers and/or with nucleic acids. This results in the formation of stable covalent bonds between MDA and the above

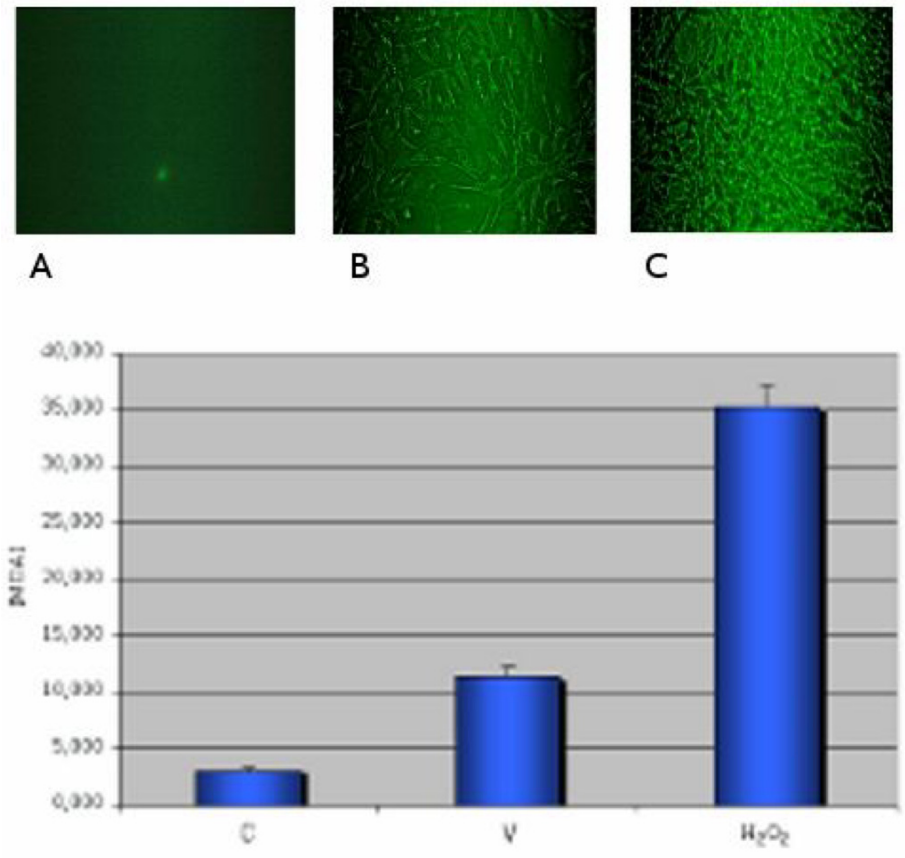

Figure 7: TUNEL and Lipo-peroxidation assay for the evaluation of cell death vesicle-treated cells. Upper Row: Left panel: Untreated cells. Center Panel: Cells exposed to 35 μM vesicles for 24 hours. Right Panel) Cells treated with H_2O_2 as positive control of membrane lipo-peroxidation. In the center panel B an evident DNA fragmentation is monitored as compared to the untreated cells (left panel A). DNA fragmentation occurs both at single strand nicks and double helix breakages; this is considered a specific sign of cell death by apoptosis [75]. Lower Row: Production of MDA in cells (3T6) treated with vesicles for 4 hours at 75 μM (Bar V). Non-treated cells were the negative control (Bar C). The intracellular production of MDA is related to oxidative cell stress at membrane level [76-78]. Comparison between the effect of H_2O_2 and vesicles is merely qualitative. The errors bars indicate the Standard Error of the mean.

mentioned chemical groups [76-78]. This eventually determines a loss of membrane fluidity which constitutes the basis of its functional deficit. Incidentally, alterations of the membrane fluidity have been observed after treatment of cultured cells with a variety of potential stress inducing agents such as liposomes, non- to mildly cytotoxic natural compounds and viral infectors [79,80]. As reported in Figure **7**, the intracellular concentration of MDA in vesicle-treated cells is about two-fold higher as compared to control ones. Therefore the treatment with vesicles causes a serious oxidative stress, possibly with a consequent physical damage at the membrane level which ends in the activation of the apoptotic pathway.

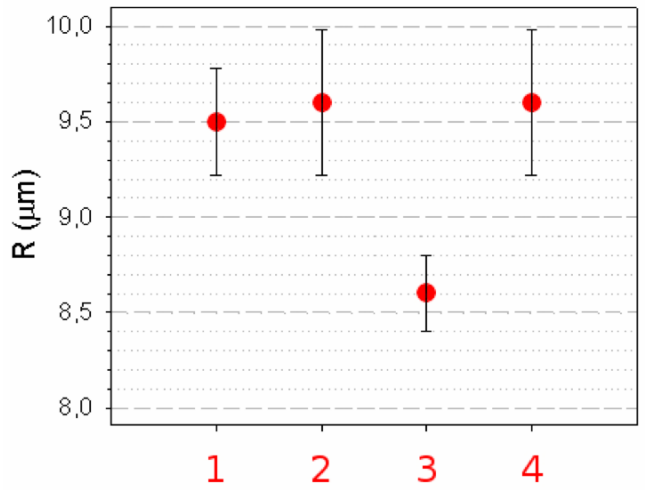

Figure 8: Histograms reporting the effect on the cell radius. The numbers indicate the variation of cell radius after treatment of cells with natural liposomes; numbers 3 and 4 letters refer to cat-anionic liposomes. Treatment time of cells was 4 hours. The highlighted area shows the statistical variation of the radius of untreated cells whose measured average radius is 8.5±0.3 μm. For further details on the experimental procedures and data analysis see Ref. [55].

The activation of this process can be actually evaluated by assessing three main hallmarks of apoptotic death: *i*, Stimulating the expression of the enzyme poly-ADP-ribose polymerase (PARP) [74] *ii*, Mitochondrial release of cytochrome c; [81] and, *iii*, Expression of caspases, cysteine-aspartic acid proteases, directly involved in the triggering and execution of the apoptotic cascade [82].

A brief description of the role, molecular function, and significance of these three hallmarks is here provided for the reader less familiar with the cell/molecular biology world. The nuclear enzyme PARP is activated following DNA damage and is commonly utilized as diagnostic of apoptotic progression. PARP is also one targets of the proteolytic cleavage operated by caspases [74,82]. The release of cytochrome c from mitochondria is also the signal that unleashes the apoptotic progression. Data from our laboratory suggest that treatment of cells with SDS-CTAB vesicles results also in a higher permeability of the mitochondrial membrane, thus causing the release of cytochrome c in the cytoplasmic matrix (please refer to the alterations of membrane fluidity shortly examined above). A further support to the idea that exposure to SDS-CTAB vesicles activates the apoptotic pathway is shown by PCR amplification of specific DNA markers. In particular, the expression of Bcl-2 gene is drastically reduced in cells exposed to cat-anionic vesicles. This gene codes for an anti-apoptotic protein located at the membrane level where it prevents the cytoplasmic release of death factors. This means that the treatment of cells with SDS-CTAB vesicles stimulates the expression of the caspases which cleave and inactivate PARP. In parallel, the release of cytochrome c and repression of Bcl-2 occur. The end of the biochemical *saga* implies a failure of DNA repair, release of death signals (cytochrome c) and deficient expression of anti-apoptotic factors (Bcl-2). The final result of these complex and intertwined biochemical processes eventually leads to progression of the apoptotic process as previously reported by our laboratory [15]. The final result of this complex and inter-twined biochemical processes eventually leads to progression of the apoptotic pathway. In conclusion, the data strongly suggest that exposure to SDS-CTAB vesicles is a primary cause of membrane damage thus causing cell death *via* activation of the apoptotic pathway. For details about the intriguing concatenation of biochemical/cellular events see the references [62,67].

Short-Term Reversible Membrane Damage and Cell Death: Making Ends Meet

Cat-anionic vesicles do show cytotoxic action at relatively high concentrations and interestingly, they are more toxic towards human tumour cells than normal stabilized murine fibroblasts. This effect, as mentioned above, may be explained by an intrinsic different membrane permeability of tumour cells with respect to normal ones, as also discussed in the “classical” works by Van Blitterswijk and Shinitzki [63-65]. In any case, the data discussed so far allow concluding that the cell membrane is possibly the main target of SDS-CTAB vesicles. This emerges from the membrane lipo-peroxidation assays in which the main product of oxidative stress, MDA, is significantly increased in vesicle-treated cells. The level of DNA damage, the levels of death markers as well as the

higher permeability of the mitochondrial membrane, lead to the conclusion that cell death occurs *via* the activation of the apoptotic pathway.

However, recent data from our laboratory showed that, at least in the case of administration of liposomes to cultured cells, a null to moderate death was observed [55,56]. In the following we shall settle this apparent discrepancy.

The data were obtained by a powerful biophysical strategy known as electro-rotation (ER) [83,84]. The latter allows single cell analysis and has been successfully used in a number of different biological systems. This technique has been illustrated and critically discussed in previous works from our and other laboratories [55,56,66,79,80,85-92]. The results of electro-rotation measurements carried out on cells exposed for 1 hour to DMPC/Gemini liposomes show that the interaction with these liposomes causes a significant variation of the dielectric parameters of the plasma membrane. In particular, a drastic reduction of the membrane properties directly related to membrane function/structure, i.e. the specific capacitance C and conductance G, is observed. This reduction is also associated to an increase of the average cell radius [55]. However, changes of the dielectric parameters apparently do not affect the cell viability and are in general the result of different concomitant causes, reflecting changes in membrane organization, ionic permeability and membrane thickness. In this context, it is worth to note that the "membrane thickness" must be interpreted as an average or "effective" thickness of the "membrane domain" [91], which takes into account the membrane micro-villosity [93]. An alteration of the bi-layer thickness could imply a rearrangement of the membrane protein moieties within the lipid matrix, and a consequent impairment of the protein function [94]. However, the observed changes do not interfere significantly with cell functionality; this fact suggests that they are ascribed mainly to a variation of the effective membrane thickness or, in other words, to changes of the membrane roughness and sinuosity. In any case, the data synthetically reported here imply that the effects of trans-membrane penetration by liposomes, and possibly by other forms of supra-molecular aggregates such as vesicles, are of transient origin since both cell size and membrane function are restored upon medium term exposure to liposomes (Figure **8**). Finally, it should be noted that in the experiments with cat-anionic vesicles, the time of treatment was far longer than in the case of liposomes. Therefore, a membrane damage possibly not directly associated to exposure to cat-anionic vesicles may become evident.

Transfection of Reporter mRNA and Protection from RNase Hydrolytic Attack

One the final aims of our work was to investigate the expression of nucleic acid after transfection into recipient cells. To this purpose a good tool consists in measuring the intracellular level of the enzyme Chloramphenicol-Acetyl-Transferase (CAT). The cognate mRNA of this bacterial enzyme can be translated into active protein in eukaryotic cells. The rationale of these experiments is that CAT is not normally present in higher cells and can therefore derive only from the biosynthetic processing of bacterial mRNA. Therefore, detection of this enzyme clearly signals that the CAT-mRNA has been successfully transferred across the plasma membrane by the vesicles and, subsequently, translated into protein within the cytoplasm matrix. Experiments where CAT-mRNA was transfected into higher cells using vesicles as vectors allow the quantification of the intracellular concentration of the enzyme by the ELISA immuno-enzymatic assay [94]. If the CAT-mRNA is added to pre-formed vesicles, the efficiency of RNA intracellular delivery and translation mediated by is lower as compared to commercially available liposome transfection systems, Figure **9**. One can reasonably expect that RNA is anchored *via* electrostatic interactions to the outer vesicle surface, where the cargo molecule becomes an easy target for hydrolysis by resident RNases. In fact, messenger RNA exists in a quasi-linear molecular configuration, which is easily prone to nucleolytic attack. This is validated by the transfection of naked CAT mRNA which is almost totally hydrolyzed by these RNases. This is shown by the almost total absence of immuno-reaction between the CAT-protein and anti-CAT antibody in the case of naked CAT-mRNA.This fact is consistent with the idea that RNA becomes unavailable to be translated into protein since it is demolished by the cytoplasmic RNases. When vesicles are formed in the presence of CAT-mRNA, the situation changes drastically. In this case, it is expected that RNA would be hosted in the aqueous lumen internal to the vesicles; therefore in the lipo-plexes thus obtained the RNA is protected by the nucleolytic attack which results in an improved delivery of the cargo molecule. The result is a significant increase of the transfecting performance of the vesicles and strongly suggests that RNA is internalized and protected within the vesicle.

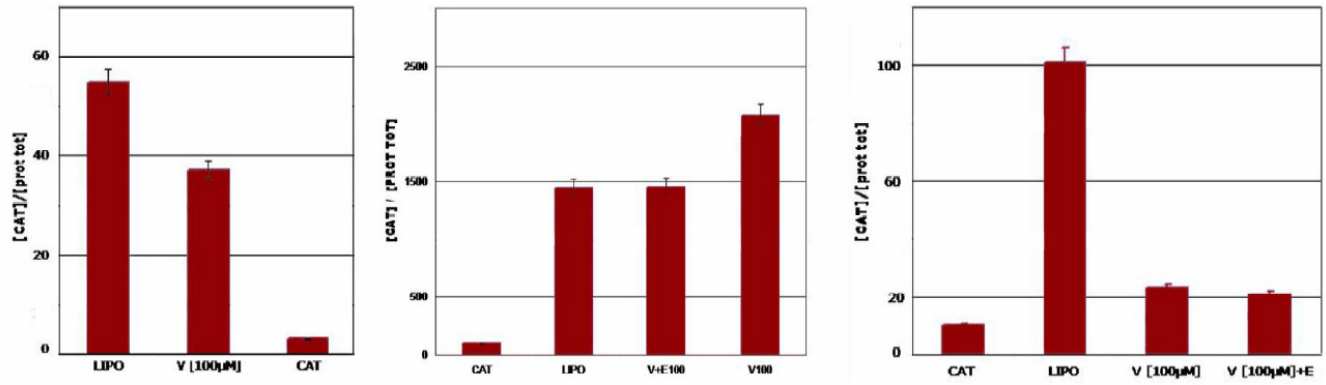

Figure 9: Transfection efficiency of mRNA-CAT of vesicles formed in the absence or presence of RNA and effect of freezing. mRNA-CAT was added to pre-formed vesicles (Left panel) or to surfactant mixture (Center Panel). In the first case the intracellular level of CAT is lower for vesicles (central bar) than Lipofectamine™ (commercial transfection system, left bar). When the CAT-mRNA is added to the surfactant mixture its expression increases significantly. Therefore the cargo molecule, internalized within the vesicle aqueous space is protected by the nucleolytic attack. Vesicles formed in the presence of mRNA-CAT and kept frozen at -20 °C for 24 hours (Right Panel). After thawing, samples were treated with RNase and transfected into HEK-293 cells. The Rnase treatment almost abolishes the translation of CAT mRNA into protein. LIPO (Lipofectamine) V[100 µM] (100 µM vesicle concentration); CAT (naked CAT mRNA)); V+E (Vesicles + RNase); V (Vesicles not treated with RNase); CAT (naked CAT mRNA).

One final question is: how does the storage temperature influence the transfection performances of vesicles? Previous evidence from our laboratory implicates that vesicles are quite stable in a temperature range of 15-25 °C [67,95]. Therefore, it becomes interesting to analyze whether freezing may damage the molecular integrity of the vesicles, thus influencing their transfection efficiency. The final conclusion is that the freezing disrupts the supra-molecular organization of the vesicles, thus abolishing their role as potential machines for the delivery of bioactive polymers.

FINAL REMARK

The research field on nano-particles such as vesicles, liposomes, nanotubes and graphene has been greatly expanding throughout these last years. In spite of the potentials of these nano-"carriers" they remain in the domain of basic research although some applicative work has been attempted at least with cultured cell models of various types [6,7,66,95]. An extremely attractive possibility is constituted by the combined application of nano-particles and medical application such as tissue engineering and repair/regeneration of damaged tissues. In any case the investing in the field of biomaterials and tissue repair/regeneration represents the future development of basic research applied to the every day necessities of bio-medical disciplines and life sciences in general. A very suggestive example is represented by the possibility of regenerating cells of the central nervous central system or bone reconstruction. This latter aspect is of particular interest a for astronauts and space voyagers whose skeleton system undergoes, among other phenomena, loss of calcium that reduces dramatically their quality of life. In this context the use of natural and/or synthetic gels/hydrogels and the development of solid substrates and scaffolds will play a crucial role. For updated works where these issues are dealt with in detail, please refer to pertinent references [96-99]. One final consideration is that these works, including the one developed in our laboratory and presented in this review article, stem from a multidisciplinary strategy for the attainment of the results. In our opinion, this is the correct approach, *i.e.* cooperation between scientists with very diverse expertise, to reach stimulating and fruitful results.

CONCLUSIONS

This work represents an inter-disciplinary effort to combine the biophysical and chemical background of vesicles preparation and structural/functional study with the analysis of their potential use in biomedical applications. The results discussed in this contribution clearly indicate a very close correlation between the structural organization of surfactants to form vesicular carriers and their biological performances. The efficiency to bind biopolymers is directly related to size and surface charge density of the vesicles illustrated here. In any case, the main contribution to the binding efficiency is due to electrostatic effects: this enables these supramolecular aggregates to achieve a good stability after formation of lipo-plexes carrying the cargo molecule (we have discussed here only the case of a

specific bacterial messenger RNA coding for the enzyme Chloramphenicol-Trans-Acetylase commonly known as CAT). The substantial possibility of the release of this molecule from vesicles, when the latter are internalized into cells, has also been discussed in this review. A preliminary, but most critical aspect is represented by the evaluation of the biocompatibility (null to minor cytotoxicity) of these vesicles. Toxicity can be modulated, as a matter of fact, by changing the surfactants or lipids moiety to be used in the preparation or modulating their dose and time of treatment of cultured cells.

The results obtained indicate that the cell membrane is possibly the main target of the vesicles under discussion (SDS-CTAB, mainly). The level of DNA damage and that of death markers, as well as the higher permeability of the mitochondrial membrane, imply that cell death occurs *via* the activation of the apoptotic pathway. This conclusion is supported from the membrane lipo-peroxidation assays in which the main product of oxidative stress, MDA, is significantly increased in vesicle-treated cells. However it should be pointed out that these cytotoxic effect are monitored after relatively long treatment times and at high concentration. Furthermore, recent data from our laboratory, obtained with natural and cat-anionic liposomes,demonstrate that these effects on the plasma membrane have a short-term character and are reversible. In any case, the effects of the vesicles are monitored at relatively high concentrations. The possibility of using supra-molecular aggregates in nano-biotechnology for the delivery of diverse molecules, therefore, remains still open.

The interaction of CAT-mRNA with the vesicles causes its internalization within the supra-molecular aggregate. To our knowledge, this is one of the first examples of an mRNA being delivered within a cell and translated into protein with a properly folded conformation: this is shown by the experiment of RNA protection. The ELISA approach in fact evidences the interaction antigen/antibody (CAT-protein/antiCAT antibody) only if the antigen is in the correct and presumably active molecular conformation.

A final interesting aspect is the mode of cell death consequent to exposure of cells to vesicles at medium to high dosages and for prolonged time. Evidence from our laboratory indicates that the administration of vesicles to cultured cells causes apoptosis, which is a multistep and very complex mode for a cell to die. The elucidation of the key steps in this process may help the investigators engaged in this field, to set up the best experimental protocols where, airrelevant cell mortality coincides with an optimal delivery of the cargo molecule of nano-biotechnological interest.

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