7

Cat-anionic vesicle-based systems as potential carriers in Nano-technologies

Aurelio Barbetta[1], Camillo La Mesa[1], Laura Muzi[2], Carlotta Pucci[1], Gianfranco Risuleo[2], Franco Tardani[1]

[1]Dept. of Chemistry, and [2]Dept. of Biology and Biotechnologies , "Sapienza University of Rome", P.le A. Moro 5, I-00185 Rome, Italy.

Outline:

Introduction

Vesicular systems offer great potentialities in delivering biological macromolecules, drugs, and cosmetics across the cell membrane [1-5]. The evaluation of their bio-compatibility is important in the application of these supra-molecular structures in biotechnology. This is of fundamental relevance when treating cells, or tissues, with vesicular or other carriers [6-16]. Undoubtedly, the physico-chemical properties of these vesicular aggregates must be properly tuned for the application. They play a crucial role in the interactions between vesicles and biopolymers, and of the resulting complexes with cells. The bio-oriented aspects must be combined with biophysical and physico-chemical strategies we report on, and be supported by synthetic work.

The preparation and characterisation of lipido-mimetic systems, LMSs, promising platforms for an efficient biopolymer binding and transfection are discussed. The matrices considered take the form of vesicles capable of significant exchange of matter with cells or tissues. LMS are vesicles and/or lipo-plexes (biopolymer-vesicle complexes) with sizes in the range 100-500 nm size. Hence, are suitable for bio-oriented applications. A plausible uptake mechanism of the above adducts involves the adhesion of lipo-plexes onto cells and subsequent pynocytosis, or fagocytosis. When the biopolymer is finally transfected in the cell matrix, it will activate the required biochemical reactions. Vesicles are chaperons for biopolymer(s) transfer into cells. To ensure a real bio-compatibility, they must be excreted or recycled at the end of the process.

The unique properties of cat-anionic vesicular carriers make applications extremely promising, potentially ensure a more efficient transfection compared to micelles, inorganic solid particles, co-acervates, and so forth. They operate in a controlled way and presumably, with no (or low) toxic effects. The rationale suggesting the use of vesicular carriers with respect to other matrices [6, 13-15] is due to the combination of different factors:

- high bio-compatibility towards cells and, eventually, tissues;
- significant adsorption onto vesicles of the species to be transferred;
- tunable physical state (gel, liquid- or liquid crystalline) of the composites, very similar to that of the cells;
- substantial and efficient binding onto cells.

Cat-anionic vesicles are supra-molecular aggregates formed by mixing in non-stoichiometric ratios cationic and anionic surfactant species [17-19]. Surfactants of opposite charge tend to aggregate in polar solvents, such as water. The electrostatic interactions between the polar heads and the hydrophobic tails favor the formation of self-assembled and organized supra-molecular structures. This phenomenon depends upon the so-called “Critical Micellar Concentration” (CMC), which represents the limit above which the surfactants in solution aggregate to form spontaneously micelles with different morphologies [20]. The diverse shapes depend on the geometry of the individual surfactant molecules. The relationship between molecular geometry of the surfactant and the morphology of the self-organized structures can be determined by the packing parameter (P) [21], i.e. the ratio between the volume of the hydrophobic tract (V) and the area of the polar head (A) times the length of the chain (L) being part of the same surfactant (P=V/AL). The modulus of P suggests the type of structure/shape that surfactants tend to assume upon aggregation. Vesicles form when the packing parameter reaches an optimal value leading to the formation of a close double layer.

The preparation of stable cat-anionic vesicular systems is not fully understood as to whether their stability is of thermodynamic, or kinetic, origin. The best strategies to control their size and charge density, and conditions for an efficient biopolymer binding are also described. Data relative to a few

selected systems, recently proposed and utilized on the purposes indicated above are discussed along with details of the preparation of cat-anionic vesicles based on lipid and/or surfactants. Optimization is necessary, since tuning the related physico-chemical properties is a prerequisite.
Preliminary physico-chemical aspects to be clarified concern vesicle size, charge density, bi-layer fluidity, as well as the charge and conformational state of the biopolymer(s) to be eventually transferred to cultured cells. The focus of this work is on biopolymer adsorption, biological assessment of the resulting vesicles and/or lipoplexes, and transfection methods. For the evaluation of biological effects, the protocols required for an effective cytotoxicity screening and uptake of exogenous bio-macromolecules are described.
The interaction of vesicles with bio-macromolecules, such as DNA, RNA or proteins, results in the formation of the cited lipoplexes. Hence, the potential tool to deliver genetic material across the cell membrane upon the formation of complexes between bio-macromolecules exposing a net negative charge such is the case for DNA and RNA, and vesicles having a positive surface charge. These complexes could be potentially delivered within the cell [6, 13]. However, cell cultures exposed to the action of vesicles or lipoplexes may suffer cytotoxic dose/response effects and are sensitive to the exposure time. There is little reported work on this specific aspect [22, 23] but recent work showed that tumor cells exhibit a higher sensitivity to treatment with SDS-CTAB vesicles as compared to normal mouse fibroblasts [24]. The experimental conditions can be adjusted to obtain an efficient vesicle-mediated transfection leading to the expression of exogenous genetic material [25 - 27]. Pilot-studies suggest that cat-anionic vesicles may find a use in anticancer therapy. The cytotoxic action of both the individual surfactants and vesicles formed by such compounds on HEK-293 cultured cells are reviewed. The transfection of an exogenous RNA mediated by SDS-CTAB vesicles and the level of translation of the reporter-protein are also reported. The data presented show that the nucleic acid is translated into protein with the correct configuration since it immuno-precipitates in the presence of the specific antibody. The novelty is that naked RNA, a biomacromolecule vulnerable by the resident RNases, is protected when it is vesicle-bound. Therefore, the above systems may find a use in biotechnology and gene therapy.

Physico-chemical background

Cat-anionic systems

The first attempt to obtain cat-anionic systems date back to the eighties of last century when Wennerström suggested that stoichimetric mixtures of two oppositely charged surfactants could give rise to lamellar order similar to phospholipids [28]. Lamellar, smectic, solids were experimentally observed. The definition of cat-anionic systems took place and it should be noted that the term “cat-anionic” was originally proposed by Ali Khan [29]. It was commonly accepted that non stoichiometric mixtures were far more appealing than stoichiometric ones. Balanced microemulsions, liquid crystals, and/or vesicles made of cat-anionic species occurred depending on the components [29-31]. Whether the stability of the latter is of thermodynamic or kinetic nature is debatable: in some instances the former definition applies [32].
Cat-anionic vesicles form in mixtures made of alkyl- or dialkylammonium salts (such as cetyltrimethylammoniun bromide, CTAB [18,33-35], tetradecyltrimethylammoniun bromide, TTAB [36], didodecyldimethylammoniun bromide, DDAB [37-39], dioctyldimethylammoniun bromide, DODAB [40,41]), and alkyl sulfates, (sodium dodecylsulfate, SDS, sodium octylsulfate, SOS), sulfonates (sodium bis-2-ethylhexylsulfosuccinate, AOT, [41,42], tetraethylammonium perfluorooctansulfonate, TEAPFOS,

[36]), carboxylates (sodium perfluorohexanoate, SPFH, [43,44]). Hydrocarbon-fluorocarbon mixtures were used, but are not stable, mostly when the fluoroalkyl chain is relatively long. Also mixtures made of DDAB and bile acid salts were considered [45]. Other systems have been reported in recent literature findings [17, 46]. In particular, mixtures of amino-acid based surfactants, AABS, and oppositely charged surfactants, or phospholipids, such as DPPA, exhibit the same phase behavior as the classical cat-anionic ones reported above. Because of their very low cyto-toxicity and high bio-compatibility AABS are sensitive to the medium pH, and their polar head groups are easily hydrolyzed. In addition, size modulation is induced by titration of acid or basic groups lying on the vesicle surface. The mixtures of lipids or of single chain surfactants are in the same category. A tentative list of the species considered to date is reported in Table 7.1.

TABLE 7.1
Acronyms and symbols indicating the species used to date to prepare cat-anionic vesicles.

Cationic

Acronym	Chemical name	Ref.
CTAB	Cetyltrimethylammonium bromide	[30-32]
TTAB	Tetradecyltrimethylammonium bromide	[33]
DTAB	Dodecyltrimethylammonium bromide	[34]
DDAB	Didodecildimethylammonium bromide	[35-37]
DODAB	Dioctyldimethylammonium bromide	[38,39]
AABS14	1,2-dimyristoyl-rac-glycero-3-O-(NR-acetyl-L-arginine) HCl	[40]
AABS12	1,2-dilauroyl-rac-glycero-3-O-(NR-acetyl-L-arginine) HCl	[40]

Anionic

Acronym	Chemical name	Ref.
SDS	Sodium dodecylsulfate	[19]
SOS	Sodium octylsulfate	[30,31]
AOT	Sodium bis-2-ethylhexylsulfosuccinate	[38,39,44,45]
SL	Sodium laurate	[41]
KPFH	Potassium perfluorohexanoate	[42]
TEAPFOS	Tetraethylammonium perfluorooctansulfonate	[33]
DPPA	Sodium 1,2-dipalmitoyl-sn-glycero-3-phosphate	[40]
STDC	Sodium taurodeoxycholate	[43]

The sequence leading from spherical micelles to vesicles implies a series of intermediate steps. On increasing the concentration of the minor surfactant ion up to charge neutralization, cat-anionic mixtures follow the sequence:

spherical micelles → swollen micelles → cylindrical micelles → vesicles → precipitates

The above sequence is controlled by the [C/A] charge (and mole) ratio, where C indicates the cationic and A the anionic species.
The surfactant ions used to prepare such mixtures can be single or multiple chain ones. Mixing oppositely charged single chain surfactant ions gives pseudo-double-chain cat-anionic surfactants, that is an analogue of single lipids. Literature also deals with single-double chain surfactants, forming pseudo-triple-chain cat-anionic mixtures. Phase behavior and the characterization of the more exotic pseudo-tetra-chain cat-anionic surfactants (DDAB/AOT) systems have been reported by Caria and Khan [48], and Karukstis et al. [49], respectively.

General considerations on surfactants or lipids

Due to their molecular ambivalence [50], due to the presence of polar and strongly non-polar moieties, surfactants and lipids self-organize to minimize the respective Gibbs energy contributions to the system stability. The non polar regions assemble in a fluid state, very similar to liquid hydrocarbons. Conversely, the polar groups face toward the aqueous solvent and stabilize the resulting aggregates and micelles, vesicles and bi-layers are formed. The two parts of these molecules are located in regions of different polarity. This is a prerequisite getting organized surfactant assemblies. The process is controlled by the “hydrophobic effect”. Water is released from the alkyl chain surroundings, and the process is entropy driven [51]. Compartmentalization in regions of different polarity, therefore, is a prerequisite to attain organized surfactant assemblies.
The size of the aggregates formed is controlled by an additional constraint ensuring a preferred supra-molecular arrangement [52, 53]. The three-dimensional geometry of surface active molecules is characterized by the polar surface area, A, the volume of hydrocarbon chain(s), V, and the length of hydrophobic moieties, L, equal to the alkyl chain in extended conformation, Figure 7.1. The resulting a-dimensional V/AL ratio pertinent to a given amphiphilic molecule implies that spherical micelles ($V/AL \leq 1/3$), cylindrical micelles ($1/3 \leq V/AL \leq 1/2$), vesicles ($1/2 \leq V/AL \leq 1$), or planar bi-layers ($V/AL \approx 1$) may be formed. Modulating the geometry of the resulting aggregates is achieved by adding salts, long chain alkanols, fatty acids, sterols, and or by mixing two surface active species [54-58]. Hence, the molecules preferentially forming spherical micelles are forced from composition and other physico-chemical constraints to assume the form of disks, rods, or vesicular entities. Particularly appealing is the possibility to get by-layer vesicles. In all cases we report here, vesicle size and charge can be modulated by the mole ratio between anionic and cationic amphiphiles.

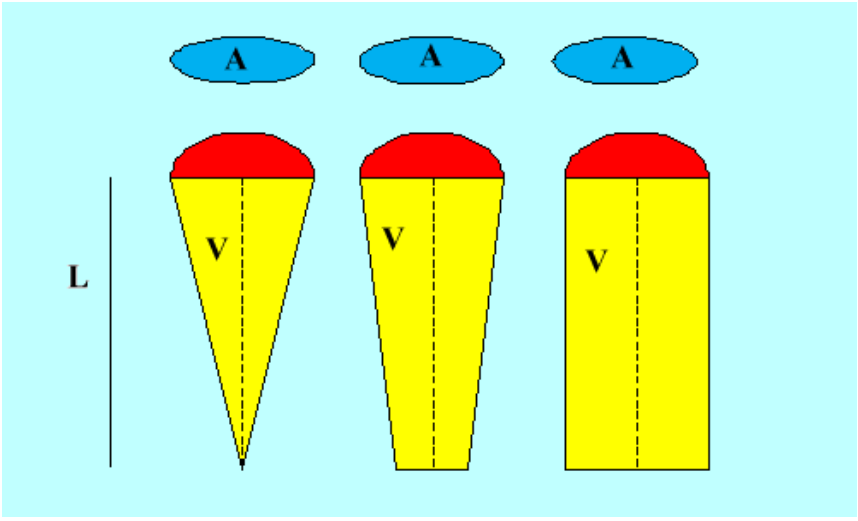

FIGURE 7.1
Two-dimensional view of surfactant molecules. The symbol A indicates the planar projection of the area per polar head group, L the alkyl chain length in extended conformation, and V the alkyl chain volume. The a-dimensional V/AL ratio pertinent to a given species implies that spherical micelles ($V/AL \leq 1/3$), cylindrical micelles ($1/3 \leq V/AL \leq 1/2$), vesicles ($1/2 \leq V/AL \leq 1$), or planar bi-layers ($V/AL \approx 1$) may be formed, in sequence. In proper conditions, the theory can be extended to surfactant mixtures, as well.

Electrostatic effects play a relevant role in the stability of such aggregates. Similarly charged groups facing outward the aggregates repel each other. To reduce such destabilizing effect counter-ions are firmly bound in the Stern layer of the aggregates [59] because of the fluid nature of such interfaces and it is also possible inserting in the aggregates oppositely charged amphiphilic species. Thus, mixing oppositely surfactants is the route to attain size and charge modulation. Hence, it is easy to tune the average interfacial curvature of the aggregates, i.e. their size.
Vesicles are characterized by sizes ranging from 10 nm to 1 μm. At fixed surfactant content, their surface charge density, σ, scales with the mole ratio and is in inverse proportion to the curvature radius, R_H [60] due to the semi-fluid nature of the bi-layers dictated by surface energy. At equilibrium, vesicle stability is controlled by the action of different forces acting on the bi-layers. Accordingly, the optimal R_H value will be tuned by the overlapping of several terms. In the case of spherical entities, in particular:

a) the surface charges increase vesicle size;
b) surface tension terms minimize its area;
c) the bi-layer curvature elasticity tends to restore the original conditions after application of deformations;
d) the optimal molecular packing, controlled by vdW forces and electrostatics, dictates the preferred vesicle size (at a given surfactant concentration);
e) the osmotic gradients active across the vesicle bi-layer may decrease, or increase, vesicle size.

The combined action of the above contributions compels the vesicle to a preferred average curvature radius, $<R_H>$. In this context, entropy contributions always play an effective role in vesicle stability. These contributions arise from an un-favored odd-even distribution of the surfactant species in the bi-layers and from other entropy of mixing terms. It is conceivable, that the composition in the inner and outer part of the bi-layer may be different, due to packing and/or curvature constraints.

At a given composition, vesicles put in contact with the solvent partition their components in such a way that the respective chemical potentials in the aggregate and in the bulk, indicated as μ_i, are the same [61, 62]. Due to the thermodynamic equilibrium, the surface active components move to/from the vesicle, in proportion to their affinity with the solvent and/or the aggregate. Surfactants characterized by short alkyl chains are preferentially distributed in the bulk with respect to long chain ones. The same applies to lipids, although the partition occurs at much lower rates.

The partition is sensitive to the working temperature, because $(\partial\mu_i/\partial T) \neq 0$ and the related entropy term become significant, leading to relevant changes in vesicle size. Hence, cat-anionic vesicles made by short alkyl chain surfactants show a moderate thermal stability. Heating implies a vesicle size rearrangement, with subsequent changes in their dimensions. Thereafter, vesicular dispersions change their appearance from turbid to milky and, then, to opalescent or bluish color. At the end of the process, vesicles retain sizes in the 300 nm range for long times, even at room temperature (Figure 7.2).

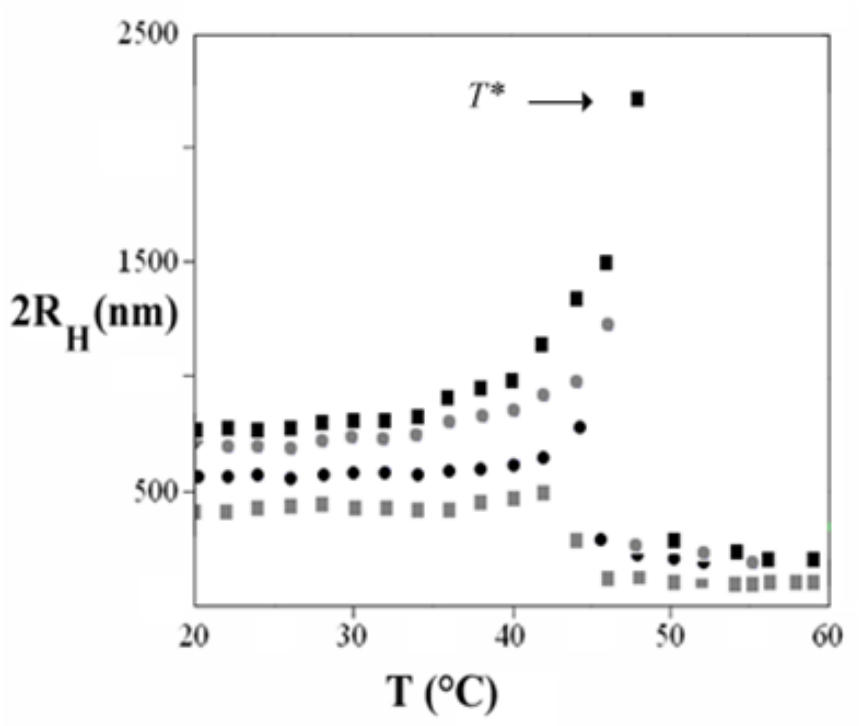

FIGURE 7.2

Average vesicle size, $2R_H$ (in nm), vs. T, in °C, for CTAB/SDS mole ratios equal to 0.286, light grey squares, 0.323, black circles, 0.379, grey circles, and 0.459, black squares. The overall mixture contains 6.0 mmol Kg^{-1} of SDS + CTAB. Note the significant increase in vesicle size on increasing T. The transition threshold depends on the CTAB/SDS mole ratios. It is considered in analogy to a second-order phase transition.

It has been demonstrated that such drastic changes in size and macroscopic appearance are concomitant to multi- to bi-layer thermal transitions [63]. The latter state is by far preferred for biomedical purposes, mainly when the internalization of a given component is required.

Vesicle preparation and characterization

As a rule, cat-anionic mixtures are formed by mixing dilute solutions (up to 10-15 mmol kg^{-1}) of oppositely charged surfactants in non-stoichiometric ratios. Mixing is rapid on an time scale (seconds) and leads to entities having sizes in the range of 10^2-10^3 nm, Figure 7.3.

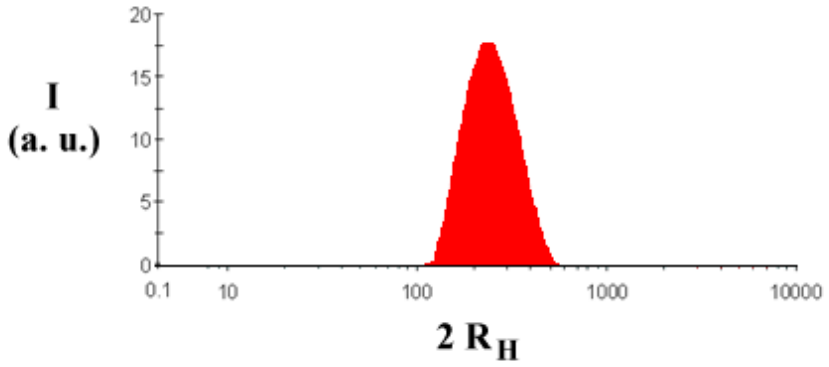

FIGURE 7.3
DLS intensity plot, showing the size distribution of a 4.05 mmol kg^{-1} DDAB/SDS vesicular system, having 3.82 as DDAB/SDS mass ratio, at 25.0°C. Note the uni-modal size distribution function.

Other approaches such as mixing the two solids and subsequent dissolving in water, or dispersing one solid surfactant into a solution of the other have also been used. Mixing of the two solutions is the fastest and more reliable route to get stable vesicles. Complete titration leads to a poorly soluble smectic (lamellar) phase, which precipitates out, leaving the respective counter-ions free in the bulk. This is a sort of metathesis. As a rule, the smectic phases obtained in this way show thermotropic, rather than lyotropic, liquid crystalline behavior [64].
Characterization by visual inspection, turbidity, DLS, ζ-potential, dielectric relaxation, TEM, surface tension, and SAXS is required. SAXS and DLS give information on vesicle size; the former also indicates the formation of bi- or multi-layer states. Cat-anionic vesicles made of SDS-CTAB are smaller than those observed in SDS-DDAB, which possibly makes them a good tool for transfection purposes. It is also worth noting that, in comparison to lipid-based vesicles, they are stable for long times.
TEM, mostly in cryo-mode, gives realistic estimates of vesicle size, bi- or multi-layer state, vesicle fusion, eventual biopolymer adsorption, formation of faceted entities and so forth [65, 66]. Atomic force microscopy, AFM, has also been used. The results are unsatisfactorily since vesicles tend to adhere, are transformed into lenses, and spread onto the surface of the substrates onto which they are deposited [67].
Zeta-potential gives information on the surface charge density, and is related to σ. Dielectric relaxation, finally, gives information on the double layer thickness surrounding the vesicles. Electro-phoretic mobility, related to ζ-potential, and interface polarization, detected by dielectric methods, jointly allow characterizing in detail the role of electrostatic contributions to vesicle stability. The results of these

measurements can be properly combined to determine the electric moment(s) active on the vesicle surface. As it is intuitive, the lower is the charge density the thicker is the double layer.
^{1}H NMR proton chemical shift measurements were also used [68]. The information is poor since the band-shapes are large and poorly resolved, Figure 7.4.

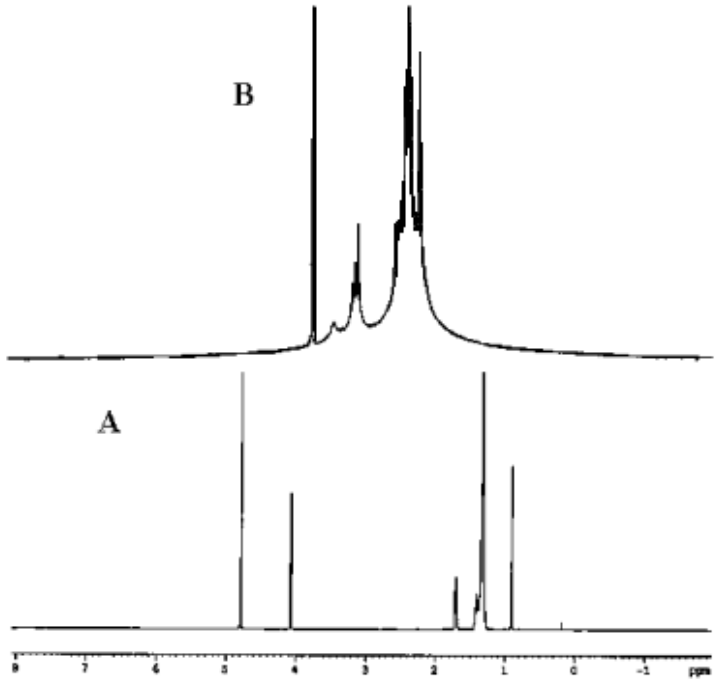

FIGURE 7.4
^{1}H NMR spectra of 20.0 mmol Kg^{-1} SDS, A, and 25.0 mmol Kg^{-1} SDS/CTAB mixture, having mole ratios 2.5/1.0. Data refer to 25.0° C. The spectra are redrawn from Ref. 74.

Conversely, NMR self-diffusion gives estimates of vesicle sizes because the decay of ^{1}H signals after the application and subsequent decay of gradient pulses is controlled by the diffusivity of the entities in which the protons are located [69, 70]. From the resulting self-diffusion values it is possible obtaining the vesicle average size, according to the Stokes-Einstein equation.
According to DLS and ζ-potential measurements there is direct proportionality between size, or charge, and the [A/C] mole ratio. When the latter is close to unity, vesicle size diverges (with eventual precipitation) and ζ-potential approaches zero, Figure 7.5.

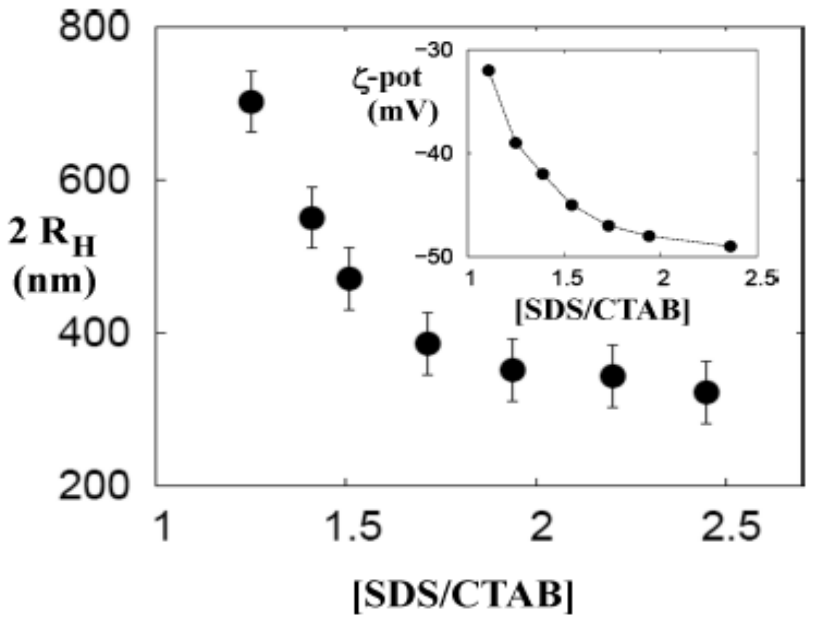

FIGURE 7.5
The average hydrodynamic radius, $2R_H$ in nm, of cat-anionic SDS/CTAB vesicles as a function of the mole (and charge) ratio, at 25.0°C. Data refer to mixtures containing 6.0 mmol Kg^{-1} as an overall surfactant content. In the inset is reported the composition dependence of ζ-potentials.

The same holds for its derivative with respect to the amount of added material. In proximity of an inflection point, derivation of the (∂ζ/∂c) function, where c is the concentration of the titrant, corresponds to (∂τ/∂c) + (∂σ/∂c) = 0, since ζ is directly proportional to στ. Therefore, the double layer thickness, τ (the Debye's screening length), diverges as the surface charge density, σ, on the shear plane of the lipo-plexes approaches zero.
The two-phase system obtained by complete titration contains a poorly soluble inner CA smectic salt, when the solution contains essentially free counter-ions. From an applied viewpoint, more interesting are the results obtained when the [C/A] mole (or charge, more precisely) ratio is ≠ 1. In such cases vesicles are formed and shown by drawing ternary or pseudo-binary phase diagrams, Figure 7.6.

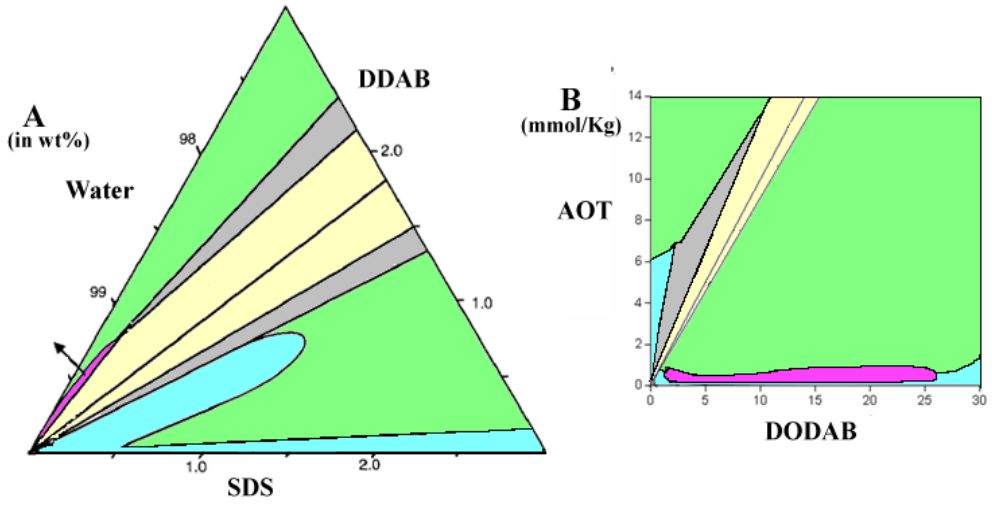

FIGURE 7.6
A. Phase map of the system water/DDAB/SDS, at 25.0° C. (A) Concentrations are in wt%. The figure has been redrawn according to Ref.s [36,37]. (B) Pseudo-binary phase diagram of the system wate/AOT/DODAB, at 25.0°C. Concentrations are in mmol Kg^{-1}. In both diagrams, the solution regions are indicated in light blue, the vesicular ones in cyan, the two phase lamellar + solution regions in green, the three phase solution + lamellar + solid in grey, the two-phase solution + crystal in yellow color. The black line dividing the yellow area in two is the equi-molar line.

Use of the latter approximation is made possible by the fact that water is always in large excess compared to all other components. The vesicular area occupies tiny regions in the phase diagram, and is usually located between the solution and the lamellar phase and/or the precipitate area. It can be readily recognized from the solutions and from the optically birefringent lamellar phase. Visual inspection, for instance, indicates the bluish, or slightly opalescent, appearance of most vesicular dispersions. The above behavior is related to the size of the disperse objects.
The region of existence is finely modulated by the overall amount of surfactant and, mostly, by their mole/charge ratios. This is a rather common feature met when preparing cat-anionic vesicles. Were the alkyl chain length the same, as in the DTAB-SDS system, the phase map would be symmetrical with respect to mole ratios, and cationic- or anionic-rich vesicles would be observed. Mixing double chain species having similar hydrophilic-lipophilic balance, such as DODAB and AOT or DDAB and AOT gives results consistent with the above statements.

Biopolymer adsorption

Small globular proteins, semi-synthetic poly-electrolytes derived from polysaccharides and/or DNA have been used as adsorbing species. As it is expected from considerations based on the molecular architecture of the species to be bound, the underlying mechanisms are quite different in the reported cases. The binding of small globular proteins, such as lysozyme and/or albumins, can be modeled in terms of the adsorption of small charged spheres onto large ones. The binding efficiency is governed by the number density of the protein with respect to vesicles and scales in proportion to the respective charges. For this to occur, it is possible to use proteins in spontaneous pH conditions. At values in the 5-

7 pH range, for instance, lysozyme has 8 positive charges in excess [68]. In such state, it promptly interacts with negatively charged vesicles by electrostatic interactions. This is the behavior observed when lysozyme is added to a dilute CTAB/SDS vesicular dispersion, in which the anionic species is in excess. Upon interaction, a sort of charge titration takes place and the size of vesicles increases. In the same time, the ζ-potential changes until surface saturation is approached. Thereafter, it remains essentially the same as the free protein. This is a clear indication that most surface sites available on the vesicle have been titrated. Therefore, it is necessary to estimate the binding efficiency. An adsorption isotherm can be drawn from electro-phoretic measurements.
Two more points still need to be considered. Saturation is not complete, due to repulsive, excluded volume, interactions between surface adsorbed protein molecules. Adsorbed lysozyme, in addition, may bridge different vesicles: such hypothesis finds support from the substantial increase in lipo-plexes size at the saturation threshold. The situation is more interesting in case of pH-dependent bovine serum albumin binding onto positively charged DDAB/SDS vesicles. The latter protein has its iso-electric point in the pH range close to 5.0-6.0. When the pH of the solution is low, no binding is observed; above pH 6.0, on the contrary, the efficiency in binding increases in proportion to the protein net charge [71], Figure 7.7.

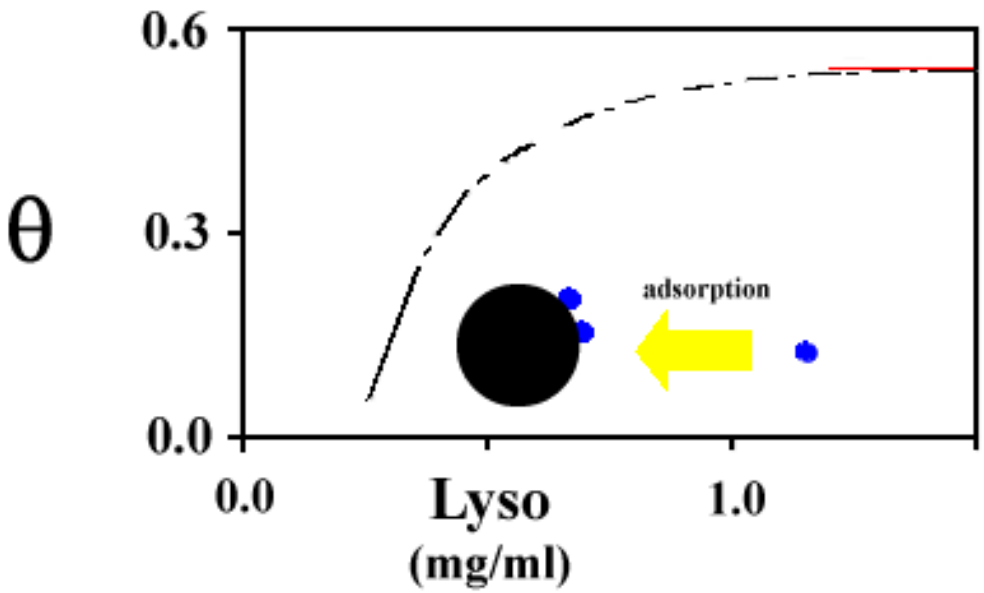

FIGURE 7.7
Surface adsorption isotherm and the related surface coverage, θ, calculated by proper rearrangement of ζ-potential values, as a function of added lysozyme, in mg/ml, to vesicular solution. Data refer to a 6.0 mmol Kg^{-1} SDS/CTAB vesicular mixture of mole ratio 2/1, at 25.0°C. Note that surface saturation occurs at high protein content, as indicated by the red line in the upper right side of the figure. Redrawn from Ref. [74].

Surface coverage changes with pH, although the number of charges neutralized upon binding remains essentially the same because more negative charges on the protein titrate a high number of surface binding sites. In addition, the interactions with vesicles is consistent with an increase in the amount of

β-sheet and random coil conformation of albumin. In such conditions, perhaps, albumin retains a significant part of its biological activity.
DNA binding, finally, has been utilized in the case of CTAB/SOS and DDAB/SDS vesicles [72, 73]. The latter bio-macromolecule is a long relatively rigid rod, which rolls around the vesicle surface. When adsorbed it does not interact with ethidium bromide. Conversely, when completely released from vesicles it has significant interactions with that dye and also retains its classical B conformation. In words, vesicle-bound DNA is substantially not accessible to the fluorophore, when is accessible to it when released. The same holds, very presumably, for all other molecules involved in stacking interactions. Released DNA reacts promptly with the products with which it is expected to interact, when it is internalized into cells.

Biomolecular and cellular evaluation of Cat-Anionic vesicles in Nano-technology

Before embarking in a study of the potential use of vesicles and similar supra-molecular aggregates in nano-biotechnology, an evaluation of their impact upon living cells is mandatory. The simplest way to investigate the effects of the exposure to vesicles is using cultured cell systems. The first effect that should be examined is the level of cytotoxic action exerted by vesicular suspensions. It should noted that the cytotoxicity is a unique feature for each vesicle type and depends upon on their chemical composition. Furthermore, the cellular/molecular phenomena underlying the toxic effect and subsequent cell death should also be highlighted. We report on cell death, on the possible mechanisms of DNA damage at the basis of this phenomenon and on the nature of the cell death. It is known that a cell dies following essentially the pathways of apoptosis and/or necrosis, even though phenomena such as necroptosis and autophagy are raising increasing interest. All the facets of these modes of cell death have been extensively reviewed in literature [74-78]. The possibilities of specific mRNA, a fundamental nucleic acid in the process of gene expression and protein bio-synthesis, once incorporated into vesicles can be successfully and efficiently transfected into recipient cells are discussed.

Evaluation of vesicle cytotoxicity and their individual components: involvement of DNA damage

The cytotoxic action of the individual surfactants SDS, CTAB and DDAB is exerted at differential extent on HEK293. The CTAB component is more toxic than SDS (Fig. 7.8, Left panel). The mortality rate is directly proportional to the concentration for both surfactants. Unpublished data from our laboratory showed that DDAB is *per se* dramatically more toxic than CTAB. Analysis of the cyto-toxicity of two different vesicles species: SDS-CTAB and SDS-DDAB, (Fig. 7.8, Center panel) shows that the latter ones are far more toxic than the SDS-CTAB ones. It is plausible to ascribe this higher toxicity to the intrinsic noxious action of DDA, although it cannot be ruled out that the association of the two chemical species may play a synergistic role. In any case, SDS-CTAB vesicles, due to their lower toxicity as compared to SDS-DDAB, are better candidates for the delivery of biological macromolecules and/or small molecules of industrial and biomedical interest. The cytotoxicity of SDA-CTAB vesicles with bound RNA results in a slight increase in cell mortality (Figure 7.8, Right panel): this may be ascribed to the commonly accepted toxic effect of free, non-vesicle associated, RNA present in the mixture.

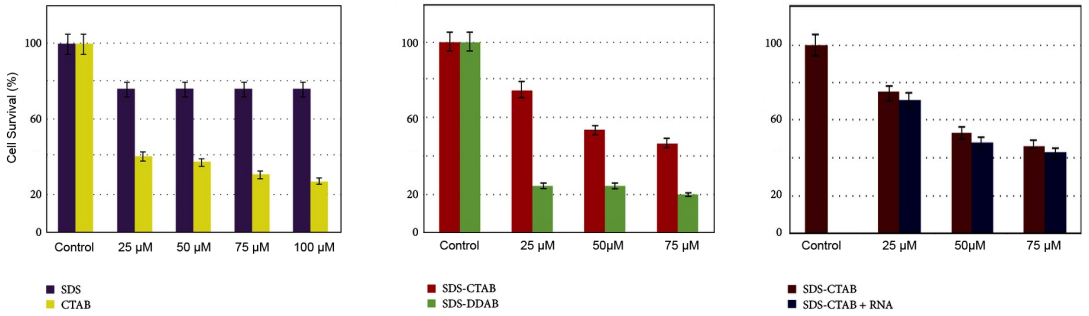

FIGURE 7.8
Effect of the separate surfactants and vesicles on the viability of HEK-293 cells. Left panel: Purple bars show the toxic effects of SDS and, CTAB (yellow bars). Center panel: Compared cytotoxicity of vesicles formed with SDS-CTAB (red bars) and SDS-DDAB (green bars). (Right panel) Cytotoxicity of vesicle/RNA lipoplexes. In all cases: Cell viability was assessed by the colorimetric Mossman assay [79]. The error bars indicate the Standard Error of the Mean.

The toxic effect of cat-anionic vesicles is dose and time-dependent as evidenced by the time course of the exposure time (Figure 7.9, Upper and Center Panel, respectively). Shorter treatment times with the vesicles do not significantly affect the cell survival. Interestingly, human tumor cells lines, such as HL60 and HeLa, are in general more sensitive as compared to the normal murine fibroblast line 3T6 (Figure 7.9 of the bottom panel). This phenomenon can be rationalized on the basis of the different structure membrane of tumor cells as compared to the normal one. Permeability may also play a crucial role since the virtual intracellular concentration of vesicles, in the case of tumor cells, could be higher than in normal ones (See also the following section for a detailed discussion of the possible role of the plasma membrane fluidity) [24]. Finally, at low concentration tumor cells do not respond significantly to the treatment thus suggesting that this population is not homogeneous but includes an intrinsically more resistant sub-population.
It is interesting to acsertain whether cytotoxicity is directly involved in a possible damage at DNA level. As shown by TUNEL assay [24], DNA undergoes a severe fragmentation in vesicle-treated cells thus suggesting a significant DNA damage which is visualized by fluorescent labelling both at single strand and double helix level (Figure 7.10, Center panel).

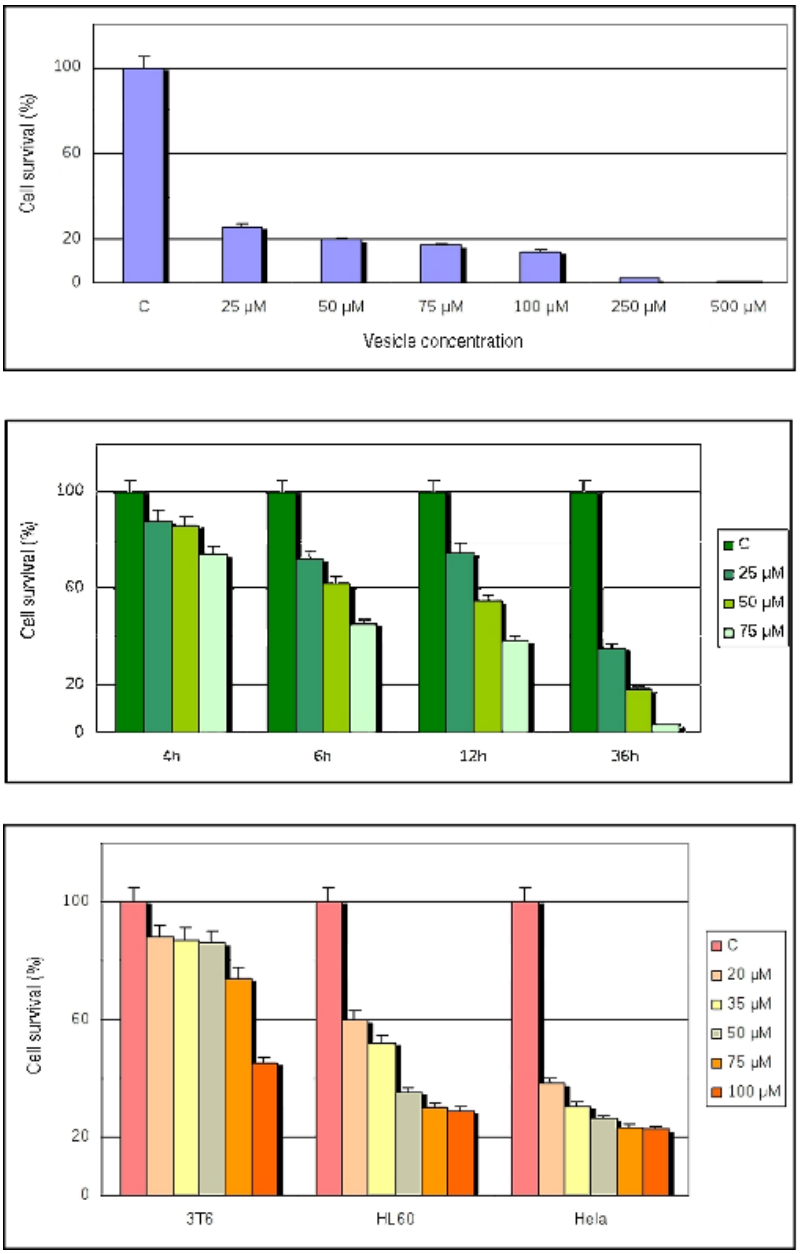

FIGURE 7.9

Dose, Time, and Differential Sensititvity to SDS-CTAB vesicles. (Upper Panel) Cytotoxity of vesicles at 24 hours of treatment. SDS-CTAB vesicles show a pronounced cytotoxicity eve at a concentration as low as 25 μM. (Center Panel) Effect of concentration and time dependence of vesicles ctyotoxicity. Murine fibroblasts 3T6 were grown in the presence of vesicles at the indicated concentration and time. (Bottom Panel) Cytoxicity 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). The cell types and vesicle concentrations are indicated in the figure. In all cases: Cell viability was assessed by the colorimetric Mossman assay [79]. The error bars indicate the Standard Error of the Mean.

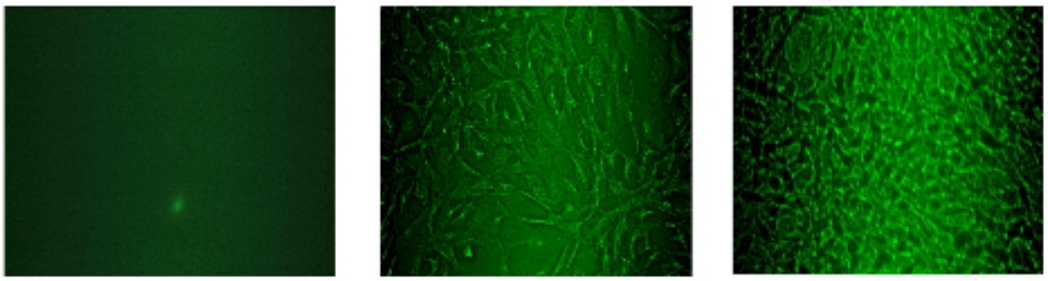

FIGURE 7.10
TUNEL assay for the evaluation of dell death. (Left panel) Untreated cells. (Center Panel) Cells (3T6) exposed to 35 μM vesicles for 24 hours. (Right Panel) Cells treated with H_2O_2 (positive control). In panel B an evident DNA fragmentation indicated by the specific fluorescent reaction, which is a sign of cell death, is present with respect to the untreated cells.

This is consistent with the cytotoxicity data discussed above: as matter of fact a heavy DNA damage may become not compatible with cell survival. However, the combined data of the toxicity tests, measured by the MTT assay [79] and the TUNEL results [24, 80], do not rule out the possibility that a defective proliferation, rather than actual cell death, is being observed or a combination of both events. A second good candidate to ascertain the mode of cell death is represented by measuring the level of membrane lipoperoxidation which is a good diagnostic of the response to an oxidative stress damage at membrane level. The role of the plasma membrane as a target for cat-anionic vesicles emerges from studies on the biochemical alterations of the lipid bi-layer as discussed in following section.

Cell death after exposure to vesicles: role of the plasma membrane alterations and level of apoptotic markers

Malonal dihaldehyde (MDA) is a compound not present in "healthy" cells but derives from the peroxidation of the poly-unsaturated fatty acids [79 - 81]. This molecule reacts with the free amino-groups of proteins, of phospholipids and/or with nucleic acids forming stable covalent bonds that eventually determine a loss of membrane fluidity, which is the basis of its functional *deficit* [82, 83]. Incidentally, alterations of the membrane fluidity have been also observed after interaction with liposomes formed with DMPC and DMPC/gemini [84] and after treatment with a non-cytotoxic natural compound [85] as well as after viral infection [86]. As shown in figure 7.11, the intracellular concentration of MDA in vesicle-treated cells is about two-fold higher as compared to controls, therefore the treatment with vesicles causes a serious oxidative stress with consequent damage at membrane level.
Combined data of the cytotoxic effect of SDS-CTAB vesicles, of the damage at the DNA level observed by TUNEL reaction and of the membrane lipoperoxidation, imply that the cell death observed after exposure to SDS-CTAB vesicles is essentially attributable to a membrane insult. A good tool to evaluate the role of this damage in the activation of the cell death process is provided by the assessment of three main phenomena*: i.e*. the activation of the enzyme poly-ADP-ribose polymerase (PARP), the mitochondrial release of cytochrome c and the expression of genes involved in the apoptotic process.

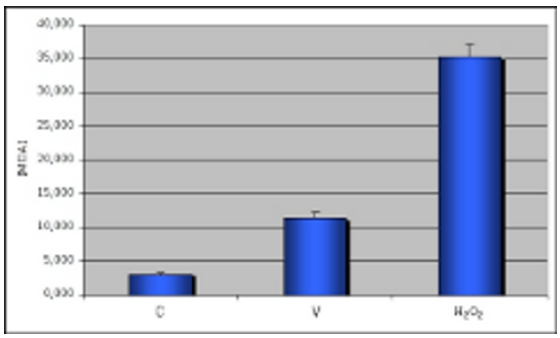

FIGURE 7.11

Lipo-peroxidation assay performed of vesicle-treated cells. Cells (3T6) were treated with vesicles for 4 hours at 75 µM. Non-treated cells were the negative control while H_2O treated cells represented the positive control. The comparison between the effect of H_2O_2 and vesicles is purely qualitative and no quantitative information can be inferred. The errors bars indicate the Standard Error of the Mean.

In particular PARP constitutes an important hallmark of apoptosis. This nuclear enzyme is activated following DNA damage and is commonly utilized as diagnostic of an on-going apoptotic process. PARP is a target of the proteolytic cleavage operated by caspases, a class of cysteine-aspartic acid proteases, which play an essential role in the apoptotic process [24, 86, 87]. In untreated cells, PARP is not cleaved by caspases (thus it appears as a single band appears after immuno-blotting (Fig 7.12). In contrast, in vesicle-treated cells the immuno-reaction evidences two different bands (with molecular weight of 116 and 85 kDa, respectively), and the amount of the cleaved fragment increases with concentration of vesicles. Hence, the treatment of cells with SDS-CTAB vesicles stimulates the expression of the caspases which cleave and inactivate PARP: the end of the biochemical story is failure to repair DNA resulting in the progression of the apoptotic process.

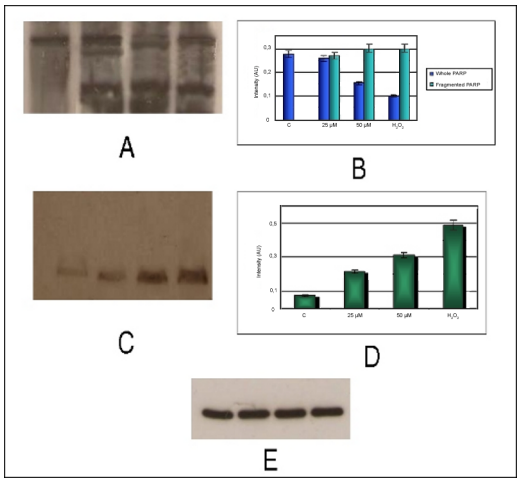

FIGURE 7.12

Evaluation of apoptotic markers. Poly (ADP-Ribose) Polymerase (PARP). Western blot pattern (Panel A) and quantitative analysis of full-length and cleaved PARP in control cells and vesicles treated cells (Panel B). Mitochondrial release of cytochrome c Western blot pattern (Panel C) and quantitative analysis of cytochrome c (Panel D). The errors bars indicate the Standard Error of the Mean. Panel E: Actin control gene.

The release of cytochrome c from mitochondria signals unleashes apoptotic progression. Our laboratory suggests that the treatment of cells with SDS-CTAB vesicles results in a higher permeability of the mitochondrial membrane, thus causing the release of cytochrome c in the cytoplasmic matrix. In any case, 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 a protein located at the membrane level where it prevents the cytoplasmic release of death factors. In conclusion, these 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.

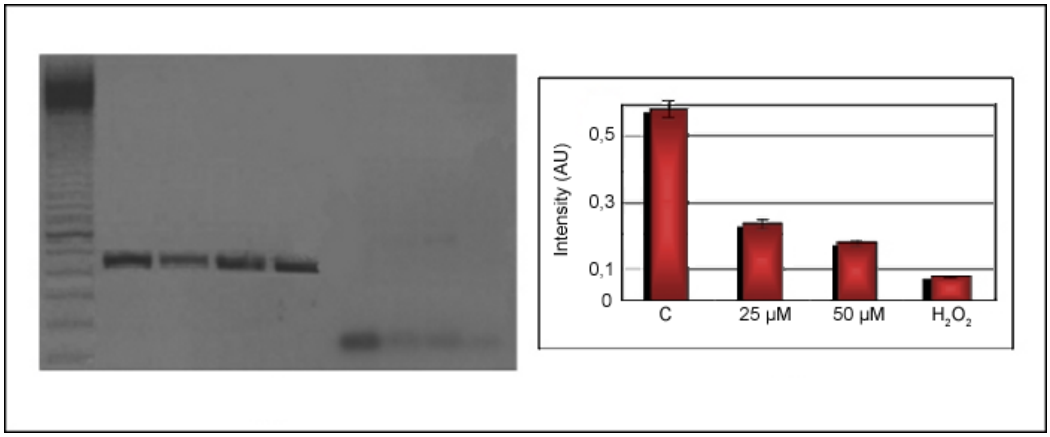

FIGURE 7.13
Amplification of the bcl-2 gene RNA by RT-PCR. (Left panel) Lanes 1 – 4: Actin control gene. Lane 5. Untreated control cells. Lanes 6 and 7: Cells treated with 25 and 50 μM vesicles, respectively. Lane 8: Cells treated with H_2O_2. (Right panel) Quantification of the Westrn blot data shown in the Left panel.

Cat-anionic vesicles do show cytotoxic action at relatively high concentrations and interestingly, they are more toxic towards human tumor cells than normal stabilized murine fibroblasts. This effect, as mentioned above, may be explained by an intrinsic different membrane permeability of tumor cells with respect to normal ones as also discussed in the "classical" works by Van Blitterswijk and Shinitzki [89 - 91]. The data discussed allows us to conclude that the cell membrane is possibly the main target of the SDS-CTAB vesicles. This emerges from the membrane lipoperoxidation 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 and the higher permeability of the mitochondrial membrane lead to the conclusion that cell death occurs via the activation of the apoptotic pathway. However, the cytotoxic effects of SDS-CTAB are monitored at relatively high concentrations, possibly above the ones normally used in pharmacological application. The possibility of using supra-molecular aggregates in nano-biotechnology for the delivery of molecules as diverse as nuclear acids, proteins and small molecules of pharmaceutical interest remains.

RNase protection assay: Transfection of Chloramphenicol-Acetyl-Transferase reporter mRNA

A powerful tool to measure the expression of nucleic acid after transfection into recipient cells is measuring the level of Chloramphenicol-Acetyl-Transferase (CAT). This is a bacterial enzyme whose

cognate mRNA can be translated into active protein in eukaryotic cells. The rationale of these experiments is that CAT is not normally present in higher cells. The detection of this enzyme is the diagnostic sign 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 HEK 293 cells, using SDS-CTAB vesicles as molecular vehicles allow the quantification of the intracellular concentration of the enzyme by the immuno-enzymatic assay ELISA. The efficiency of RNA intracellular delivery of our vesicles is apparently lower as compared to Lipofectamine, a commercially available liposome transfection system. This occurs when the CAT mRNA is added to pre-formed vesicle and one can reasonably expect that the CAT mRNA is anchored via electrostatic interactions to the surface of the vesicles and the cargo molecule becomes an easy target for hydrolysis by RNases. This observation is validated by the transfection of naked CAT mRNA is almost totally hydrolyzed by the RNases normally present in the cytoplasm. Messenger RNA exists in a quasi-linear molecular configuration, which is easily hydrolyzed by the resident RNases. Figure 7.14 shows that the immuno-reaction between the CAT-protein and anti-CAT antibody is, as expected, almost absent in the case of the transfection with naked CAT-mRNA (bar to the right); this is consistent with the idea that the RNA is demolished by the RNases present in the cytoplasm and therefore becomes unavailable to be translated into protein. The transfection efficiency at the indicated concentrations of SDS-CTAB, is indeed lower that the one exhibited by Lipofectamine (Bar to the left) but, in any case is quite satisfactory.

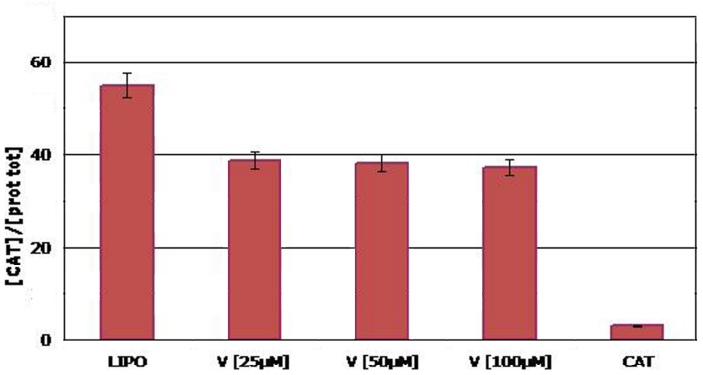

FIGURE 7.14
Transfection efficiency of mRNA-CAT. In this experiment, the RNA was added to pre-formed vesicles. The intracellular level of CAT is lower in the case of SDS-CTAB vesicles as compared to a commercial transfection system (Lipofectamine™). See text for further details. LIPO (Lipofectamine) ; V[25μM] (25 μM vesicle concentration) ; V[50μM] (50μM vesicle concentration) ; V[100μM] (100μM vesicle concentration) ; CAT (naked CAT mRNA).(Figura 5 Paper Laura).

The situation changes dramatically when the vesicles are formed in the presence of CAT-mRNA. In this case the RNA would be hosted in the aqueous *lumen* internal to the vesicles. If the lipoplexes thus obtained are transfected into the cells, the RNA is protected by the nucleolytic attack and this results in an improved delivery of the cargo molecule. Therefore a significant increase of the transfecting performance of the vesicles is monitored (Figure 7.15).

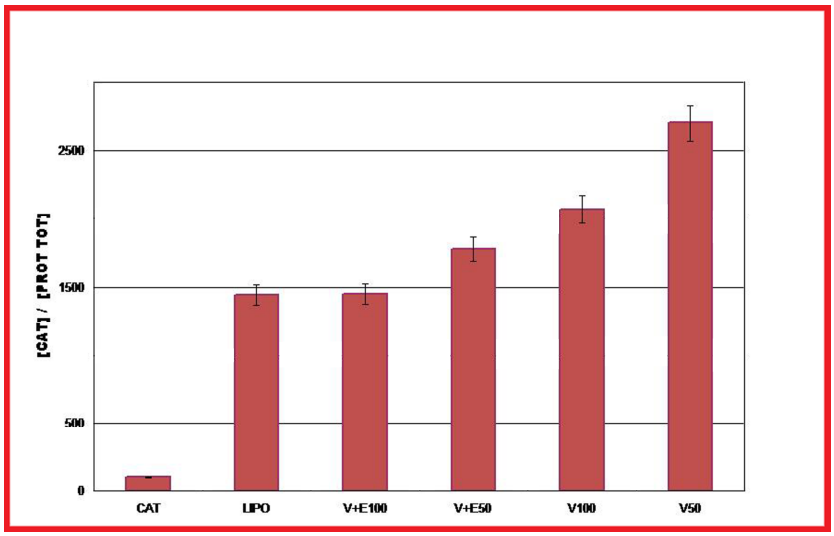

FIGURE 7.15
Transfection efficiency of mRNA-CAT of vesicles formed in the presence of RNA and after treatment with RNase. In this experiment, the mRNA-CAT was added to the surfactant mixture prior to the vesicle formation. This results strongly suggests that the RNA is internalized within the vesicle aqueous space and it thus protected by the nucleolytic attack. Interestingly, vesicles not treated with RNase (last two bars to the right) exhibit a higher efficiency than Lipofectamine.LIPO (Lipofectamine); V+E (Vesicles + RNase); V (Vesicles not treated with RNase); CAT (naked CAT mRNA). The numbers at the bottom of the bar indicate the vesicle concentration (μM).

This strongly suggests that actually the RNA molecule is internalized and protected within the vesicle. Subsequent to trasfection, the CAT-mRNA is released in the cytoplasm where is translated into protein. The last set of experiments discussed the role the storage temperature from previous evidence from our laboratory [68, 72, 73] implicates that vesicles are quite stable in a temperature range of 15-25 °C. Actually freezing damages the molecular integrity of the vesicles, thus abolishing their transfection efficiency. Freezing almost abrogates the transfection capacity of the SDS-CTAB vesicles since the translation of the CAT-mRNA drops almost to the same level as the one exhibited by the naked RNA (Figure 7.16, first bar to the left).
Therefore, it is reasonable assuming that the freezing process disrupts the supra-molecular organization of the vesicles. Consequently their role as potential molecular bio-machines for the delivery of bioactive polymers is abrogated [91].

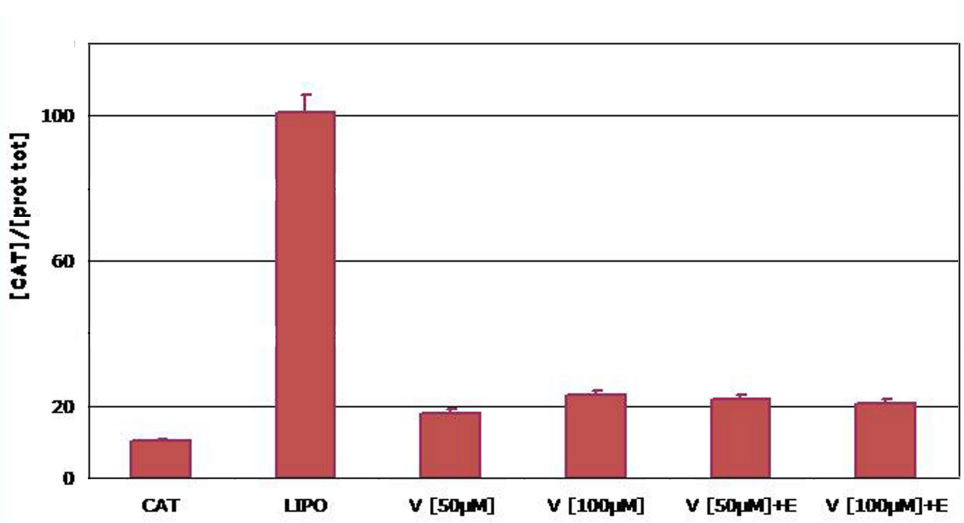

FIGURE 7.16
Effect of freezing on the transfection efficiency. Vesicles formed in the presence of mRNA-CAT and kept frozen at -20 °C for 24 hours. After thawing, the aggregates were treated with RNase and transfected into HEK-293 cells. Data clearly indicate that the Rnase treatment almost abolishes the translation of CAT mRNA into protein. LIPO (Lipofectamine); V[25μM] (25 μM vesicle concentration) ; V[50μM] (50μM vesicle concentration) ; V[100μM] (100μM vesicle concentration) ; CAT (naked CAT mRNA).

Conclusions

Data presented in this contribution clearly indicate the strict relations between the structural organization of surfactants to form vesicular carriers and the related biological performances. From a functional point of view, the efficiency in biopolymer binding is directly related to the nature of the reported vesicles, that is their size and surface charge density. The major contribution to the binding efficiency is due to electrostatic effects, which ensures good stability to the resulting lipo-plexes and substantial possibility to their release from vesicles, when the latter are internalized into cells. Toxicity can be modulated by changing the surfactants or lipids to be used in the preparation of effectively biocompatible formulations.

The results obtained indicate that the cell membrane is possibly the main target of SDS-CTAB vesicles. This emerges from the membrane lipoperoxidation 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, imply that cell death occurs via the activation of the apoptotic pathway. In any case, the cytotoxic effects of SDS-CTAB vesicles are monitored at relatively high concentrations. The possibility of using supra-molecular aggregates in nano-biotechnology for the delivery of diverse molecules, remains still open.

The interaction of CAT-mRNA with the vesicles causes its internalization within the supra-molecular aggregate. This is the first example of an mRNA being delivered within a cell and translated into a properly folded conformation as shown by the data obtained with the experiment of RNA protection.

The ELISA approach in fact evidences the interaction antigen/antibody (CAT-protein/antiCAT antibody) only if the antigen is found in the proper and presumably active molecular structure.
Finally, one interesting aspect, yet to be investigated in detail, is the mode of cell death. Previous evidence from our laboratory indicates that administration of vesicles to cultured causes apoptosis. This is a multi-step and very complex mode for a cell to die. Therefore, the elucidation of the key step(s) in the process of cell death may help the investigators engaged in this field, to set up the best experimental conditions in which, to minimal cell mortality, corresponds an optimal delivery of the cargo molecule of biotechnological interest.

Acknowledgments

We acknowledge the financial support by the Ministry of Public Education (MIUR) through a University grant to Sapienza University of Rome, Italy. We are indebted to G.A. Ranieri, C. Oliviero-Rossi and L. Coppola, at the University of Calabria, E.F. Marques, at Porto, and R. Pons, at CSIC in Barcelona, for fruitful discussions. The participation of a number of master and PhD students who actively participate to the experimental parts of this work should also be acknowledged.

Obituary

During the preparation of this manuscript we were informed that Ali Khan (formerly at Phys. Chem. 1, Lund University, Sweden), passed away. Since the end of 80's, he was one of the first scientists involved in cat-anionic vesicular systems (and in many other subjects). Ali generously shared his deep competences and collaborated with many scientists, who are honored to have been his students and collaborators. One author of this contribution (CLM) knew Ali since more than thirty years and was in friendly relations with him since that time; others knew him from his important activity in the field. We remember him friendly and dedicate this manuscript to his memory. Our condolences are for Lena, his wife, and his beloved sons Malek, Jamil, and Omar.

References

1. Richard, A.; Bourel-Bonnet, L. Internalization of a peptide into multi-lamellar vesicles assisted by the formation of an α-oxo oxime bond. *Chemistry- A European Journal*, 2005, *11*, 7315-7321.
2. Samaj, J.; Baluska, F.; Voigt, B.; Volkmann, D.; Menzel, D. Endocytosis and actomyosin cytoskeleton. *Plant Cell Monographs*, 2006, *1*(Plant Endocytosis), 233-244.
3. Liu, Y.-C.; Le N., Anne-Laure M.; Schmidt, J.; Talmon, Y.; Chmelka, B.F.; Lee, C.T. Jr. Photo-Assisted Gene Delivery Using Light-Responsive Cat-anionic Vesicles. *Langmuir*, 2009, *25*, 5713-5724.
4. Jiang, Y.; Li, F.; Luan, Y.; Cao, W.; Ji, X.; Zhao, L.; Zhang, L.; Li, Z. Formation of drug/surfactant catanionic vesicles and their application in sustained drug release*. Int. J. Pharm.*, 2012, *436*, 806-814.
5. Soussan, E.; Cassel, S.; Blanzat, M.; Rico-Lattes, I. Drug delivery by soft matter: matrix and vesicular carriers. *Angew. Chem. Int. Ed.*, 2009, *48*, 274-288.
6. Kwon, G.S.; Kataoka, K. Block copolymer micelles as long-circulating drug vehicles. *Adv. Drug Delivery Rev.*, 1995, 1*6*, 295-309.
7. Kainthan, R.K.; Janzen, J.; Levin, E.; Devine, D.V.; Brooks, D.E. Biocompatibility testing of branched and linear Polyglycidol. *Biomacromolecules*, 2006, *7*, 703-709.
8. Francis, M.F.; Cristea, M.; Winnik, F.M. Polymeric micelles for oral drug delivery: Why and how. *Pure Appl. Chem.*, 2004, *76*, 1321-1335.
9. Hawker, C. J.; Frechet, J.M.J. Preparation of polymers with controlled molecular architecture. A new convergent approach to dendritic macromolecules. *J. Am. Chem. Soc.*, 1990, *112*, 7638-7647.
10. Junping, W.; Takayama, K.; Nagai, T.; Maitani, Y. Pharmacokinetics and antitumor effects of Vincristine carried by microemulsions composed of PEG-lipid, oleic acid, vitamin E and cholesterol. *Intern. J. Pharm.*, 2003, *251*, 13-21.
11. Bhattacharya, S.; De, S.; Subramanianm M. Synthesis and vesicle formation of hybrid bolaphile/amphiphile ion pairs. Evidence of membrane property modulation by molecular design. *J. Org. Chem.*, 1998, *63*, 7640-7651.
12. Barenholz, Y.; Peer, D. Liposomes, lipid biophysics, and sphingolipid research: from basic to translation research. *Chem. Phys. Lipids*, 2012, *165*, 363-364.
13. Kocer, A. Functional liposomal membranes for triggered release. *Methods Mol. Biol.*, 2010, *605*(Liposomes, Vol. 1), 243-255.
14. Kim, S.-H.; Kim, K.-S.; Lee, S.-R.ae; Kim, E.; Kim, M.-S.; Lee, E.-Y.; Gho, Y.S.; Kim, J.-W.; Bishop, R.E.; Chang, K.-T. Structural modifications of outer membrane vesicles to refine them as vaccine delivery vehicles. *Biochim. Biophys. Acta, Biomembranes*, 2009, *1788*, 2150-2159.
15. Kahya, N.; Merkle, D.; Schwille, P. Pushing the complexity of model bilayers: novel prospects for membrane biophysics. *Springer Ser. Fluorescence*, 2008, *4* (Fluorescence of Supermolecules, Polymers, and Nanosystems), 339-359.
16. Marques, E.F.; Brito, R.O.; Silva, S.G.; Rodriguez-Borges, J. E.; do Vale, M.L.; Gomes, P.; Araujo, M.J.; Soderman, O. Spontaneous Vesicle Formation in Cat-anionic Mixtures of Amino Acid-Based Surfactants: Chain Length Symmetry Effects. *Langmuir*, 2008, *24*, 11009-11017.
17. Bonincontro, A.; Spigone, E.; Ruiz Peña, M.; Letizia, C.; La Mesa, C. Lysozyme binding onto cat-anionic vesicles. *J. Colloid Interface Sci.*, 2006, *304*, 342-347.

18. Colomer, A.; Pinazo, A.; Garcia, M.T.; Mitjans, M.; Vinardell, M. P. Infante, M.R.; Martinez, V.; Perez, L. pH-sensitive surfactants from lysine: assessment of Their Cytotoxicity and Environmental Behavior. *Langmuir*, 2012, *28*, 5900-5912.
19. Mukerjee, P.; Mysels, K.J. Critical Micellar Concentrations of aqueous Surfactant Systems. Natl. Bur. Std. Ser., *NSRDS-NBS 36*, Washington D.C. 1971.
20. Israelachvili, J.N.; Mitchell, D. John; Ninham, B.W. Theory of self-assembly of lipid bilayers and vesicles. *Biochim. Biophys. Acta, Biomembranes*, 1977, *470*, 185-201.
21. Kuo, B.J.H.; Jan, M.S.; Chang, C.H.; Chiu, H.W.; Li, C.T. Cytotoxicity characterization of catanionic vesicles in RAW 264.7 murine macrophage-like cells. *Colloids Surf. B: Biointerfaces*, 2005, *41*, 189-196.
22. Colomer, A.; Pinazo, A.; Garcia, M.T.; Mitjans, M.; Vinardell, M. P. Infante, M.R.; Martinez, V.; Perez, L. pH-sensitive surfactants from lysine: assessment of Their Cytotoxicity and Environmental Behavior. *Langmuir*, 2012, *28*, 5900-5912.
23. Cheng LC, Jiang X, Wang J, Chen C, Liu RS. Nano-bio effects: interaction of nanomaterials with cells. Nanoscale. 2013 May 7;5(9):3547-69.
24. Aiello, C.; Andreozzi, P.; La Mesa, C.; Risuleo, G. Biological activity of SDS-CTAB cat-anionic vesicles in cultured cells and assessment of their cytotoxicity ending in apoptosis. *Colloids Surf. B: Biointerfaces*, 2010, 78, 149-154.
25. Nogueira, D. R.; Mitjans, M.; Infante, M.R.; Vinardell, M.P. Comparative sensitivity of tumor and non-tumor cell lines as a reliable approach for in vitro cytotoxicity screening of lysine-based surfactants with potential pharmaceutical applications. *Intern. J. Pharm.*, 2011, *420*, 51-58.
26. Multi-compartmental oral delivery systems for nucleic acid therapy in the gastrointestinal tract. Kriegel C, Attarwala H, Amiji M. *Adv Drug Deliv Rev*. 2013 Jun 15;65(6):891-901.
27. Guo P, Haque F, Hallahan B, Reif R, Li H. Uniqueness, advantages, challenges, solutions, and perspectives in therapeutics applying RNA nanotechnology. *Nucleic Acid Ther*. 2012 *Aug*;22:226-45.
28. Jokela, P.; Jönsson, B.; Wennerström, H. Phase equilibria in a system containing both an anionic and a cationic amphiphile. A thermodynamic model calculation. *Progr. Colloid Polym. Sci.*, 1985, *70*, 17-22.
29. Jokela, P.; Jönsson, B. Phase equilibria of catanionic surfactant-dodecanol-water systems. *J. Phys. Chem.*, 1988, *92*, 1923-1927.
30. Jokela, P.; Jönsson, B.; Eichmueller, B.; Fontell, K. Phase equilibria in the sodium octanoate octylammonium octanoate-water system. *Langmuir*, 1988, *4*, 187-192.
31. Jönsson, B.; Jokela, P.; Khan, A.; Lindman, B.; Sadaghiani, A. Catanionic surfactants: phase behavior and microemulsions. *Langmuir*, 1991, *7*, 889-895.
32. Marques, E.F. Size and Stability of Catanionic Vesicles: Effects of Formation Path, Sonication, and Aging. *Langmuir*, 2000, *16*, 4798-4807.
33. Brasher, L.L.; Herrington, K.L.; Kaler, E.W. Electrostatic effects on the phase behavior of aqueous cetyltrimethylammonium bromide and sodium octylsulfate mixtures with added sodium bromide. *Langmuir*, 1995, *11*, 4267-4277.
34. Yatcilla, M.T.; Herrington, K.L.; Brasher, L.L.; Kaler, E.W.; Chiruvolu, S.; Zasadzinski, J.A. Phase behavior of aqueous mixtures of cetyltrimethylammonium bromide (CTAB) and sodiumoctylsulfate (SOS). J. Phys. Chem., 1996, 100, 5874-5879.

35. Brasher, L.L.; Kaler, E.W. A small angle neutron scattering (SANS) contrast variation investigation of aggregate composition in catanionic surfactant mixtures. *Langmuir*, 1996, *12*, 6270-6276.
36. Barbetta, A.; Pucci, C.; Tardani, F.; Andreozzi, P.; La Mesa, C. Size and Charge Modulation of Surfactant-Based Vesicles *J. Phys. Chem. B*, 2011, *115*, 12751-12758.
37. Kamenka, N.; Chorro, M.; Talmon, Y.; Zana, R. Study of mixed aggregates in aqueous solutions of sodium dodecyl sulfate and dodecyltrimethylammonium bromide. *Colloids Surf.*, 1992, *67*, 213-222.
38. Söderman, O.; Herrington, K.L.; Kaler, E.W.; Miller, D.D. Transitions from micelles to vesicles in aqueous mixtures of anionic and cationic surfactants. *Langmuir*, 1997, *13*, 5331-5338.
39. Marques, E. F.; Regev, O.; Khan, A.; Grac¸a Miguel, M.; Lindman, B. Vesicle Formation and General Phase Behavior in the Catanionic Mixture SDS-DDAB-Water. The Anionic-Rich Side. *J. Phys. Chem. B*, 1998, *102*, 6746-6758.
40. Marques, E. F.; Regev, O.; Khan, A.; Graca Miguel, M.; Lindman, B. Vesicle formation and general phase behavior in the catanionic mixture SDS-DDAB-water. The cationic-rich side. *J. Phys. Chem. B*, 1999, *103*, 8353-8363.
41. Zumpano, R.C. Cat-anionic vesicles made of dioctyldimethylammonium bromide and Aerosol OT. *Thesis*, 2013, La Sapienza University, Rome, Italy.
42. Pucci C., Salvia A., Ortore M.G.; La Mesa C. The DODAB/AOT/water system: vesicle formation and interactions with salts, or synthetic poly-electrolytes. *Soft Matter*, 2013, *9*, 9000-9007.
43. Jung, H.T.; Coldren, B.; Zasadzinski, J.A.; Iampietro, D.J.; Kaler, E.W. The origins of stability of spontaneous vesicles. *Proc. Natl. Acad. Sci. U.S.A.*, 2001, *98*, 1353-1357.
44. Iampietro, D.J.; Brasher, L.L.; Kaler, E.W.; Stradner, a.; Glatter, O. Direct analysis of SAXS and SANS measurements of catanionic surfactant mixtures by Fourier transformation. *J. Phys. Chem. B*, 1998, *102*, 3105-3113.
45. Youssry, M.; Coppola, L.; Marques, E.F.; Nicotera, I. Unravelling micellar structure and dynamics in an unusually extensive DDAB/bile salt catanionic solution by rheology and NMR diffusometry. *J. Colloid Interface Sci.*, 2008, *324*, 192-198.
46. Barran-Berdon, A.L.; Munoz-Ubeda, M.; Aicart-Ramos, C.; Perez, L.; Infante, M.-R.; Castro-Hartmann, P.; Martin-Molina, A.; Aicart, E.; Junquera, E. Ribbon-type and cluster-type lipoplexes constituted by chiral lysine based cationic gemini lipid and plasmid DNA. *Soft Matter*, 2012, *8*, 7368-7380.
47. Lozano,N.; Pinazo, A.; La Mesa, C.; Perez, L.; Andreozzi, P.; Pons, R. Catanionic Vesicles Formed with Arginine-Based Surfactants and 1,2-Dipalmitoyl-*sn*-glycero-3-phosphate Monosodium Salt. *J. Phys. Chem. B*, 2009, *113*, 6321–6327.
48. Caria, A.; Khan, A. Phase Behavior of Catanionic Surfactant Mixtures: Sodium Bis(2ethylhexyl) sulfosuccinate-Didodecyldimethylammonium bromide-Water System. *Langmuir*, 1996, *12*, 6282-6290.
49. Karukstis, K.K.; Zieleniuk, C.A.; Fox, M.J. Fluorescence Characterization of DDAB-AOT Catanionic Vesicles. *Langmuir*, 2003, *19*, 10054-10060.
50. Hartley, G.S. "*Aqueous Solutions of paraffin chain salts.*" Herman, Paris, 1936.
51. Tanford, C. "*The hydrophobic effect: formation of micelles and biological membranes.*", 2nd Ed.; 1980, Wiley, New York.
52. Israelachvili, J.N.; Mitchell, D.J.; Ninham, B.W. Theory of self-assembly of hydrocarbon amphiphiles into micelles and bilayers. *J. Chem. Soc. Faraday Trans. 2*, 1976, *72*, 1525-1568.

53. Ninham, B.W.; Evans, D.F. The Rideal Lecture: Vesicles and molecular forces. *Faraday Disc. Chem. Soc.*, 1986, *81*, 1-17.
54. Parker, A.; Fieber, W. Viscoelasticity of anionic wormlike micelles: effects of ionic strength and small hydrophobic molecules. *Soft Matter*, 2013, *9*, 1203-1213.
55. Kamaya, Hi.; Matubayasi, N.; Ueda, I. Biphasic effect of long-chain n-alkanols on the main-phase transition of phospholipid vesicle membranes. *J. Phys. Chem.*, 1984, *88*, 797-800.
56. Kokot, Z. Effect of NaCl and temperature on sodium dodecyl sulfate mixed micelles. *Chemia Analityczna*, (Warsaw, Poland), 2001, *46*, 823-829.
57. Soubeyrand, V.; Luparia, V.; Williams, P.; Doco, T.; Vernhet, A.; Ortiz-Julien, A.; Salmon, J.-M. Formation of micelles containing solubilized sterols during rehydration of active dry yeasts Improves their fermenting capacity. *J. Agric. Food Chem.*, 2005, *53*, 8025-8032.
58. Srinivasan, V.; Blankschtein, D. Effect of Counterion Binding on Micellar Solution Behavior: 1. Molecular-Thermodynamic Theory of Micellization of Ionic Surfactants. *Langmuir*, 2003, *19*, 9932-9945.
59. Stigter, D.; Dill, K.A. Free Energy of electrical double layers: entropy of adsorbed ions and the binding polynomial. *J. Phys. Chem.*, 1989, *93*, 6737-6743.
60. Zasadzinski, J.A.; Jung, H.-T.; Coldren, B.; McElvey, C.; Kaler, E.W. The origins of stability of equilibrium vesicles. *221st ACS Natl. Meeting,* San Diego, CAL, U.S.A., 2001, COLL. 053.
61. Safran, S.A.; Pincus, P.A.; Andelman, D.; MacKintosh, F.C. Stability and phase behavior of Mixed surfactant vesicles. *Phys. Rev. A*, 1991, *43*, 1071-1078.
62. MacKintosh, F.C.; Safran, S.A. Stability and phase behavior of mixed-surfactant vesicles. *Mater. Res. Soc. Symposium Proc.*, 1992, *248* (Complex Fluids), 11-21.
63. Andreozzi, P.; Funari, S.S.; La Mesa, C.; Mariani, P.; Ortore, M.G.; Sinibaldi, R.; Spinozzi, F. Multi- to Unilamellar Transitions in Catanionic Vesicles. *J. Phys. Chem. B*, 2010, *114*, 8056-8060.
64. Silva, B.F.B.; Marques, E.F. Thermotropic behavior of asymmetric chain length catanionic surfactants: The influence of the polar head group. *J. Colloid Interface Sci.*, 2005, *290*, 267-274.
65. Regev, O.; Backov, R.; Faure C. Gold nanoparticles spontaneously generated in onion-type multilamellar vesicles. Bilayers particle coupling imaged by cryo-TEM. *Chem. Mater.*, 2005, *16*, 5280-5285.
66. Marques, E.F.; Regev, O.; Khan, A.; Lindman, B. Self-organization of double-chained and
a. pseudodouble-chained surfactants: counterions and geometry effect. *Adv. Colloid Interface Sci.*, 2003, *100-102*, 83-104.
67. Bordi, F.; Cametti, C.; Diociauti, M.; Gaudino, D.; Gili, T.; Sennato, S. Complexation of anionic polyelecrolytes with cationic liposomes: evidence of reentrant condensation and lipolexes formation. *Langmuir*, 2004, *20*, 5214-5222.
68. Letizia, C.; Andreozzi, P.; Scipioni, A.; La Mesa, C.; Bonincontro, A.; Spigone, E. Protein Binding onto Surfactant-Based Synthetic Vesicles. *J. Phys. Chem. B*, 2007, *111*, 898-908.
69. Stilbs, P. Fourier transform pulsed-gradient spin-echo studies of molecular diffusion. *Progr. NMR Spectrosc.*, 1987, *19*, 1-45.
70. Stejskal, E.O.; Tanner, J.E. Spin diffusion measurements: spin echoes in the presence of a time dependent field gradient. *J. Chem. Phys.*, 1965, *42*, 288-292.
71. Pucci, C.; Scipioni, A.; La Mesa, C. Albumin binding onto synthetic vesicles. *Soft Matter*, 2012, *8*, 9669-9675.

72. Bonincontro, A.; La Mesa, C.; Proietti, C.; Risuleo, G. A Biophysical Investigation on the Binding and Controlled DNA Release in a Cetyltrimethylammonium Bromide-Sodium Octyl Sulfate Cat-Anionic Vesicle System. *Biomacromolecules*, 2007, *8*, 1824-1829.
73. Bonincontro, A.; Falivene, M.; La Mesa, C.; Risuleo, G.; Ruiz Peña, M. Dynamics of DNA Adsorption on and Release from SDS-DDAB Cat-Anionic Vesicles: a Multitechnique Study. *Langmuir*, 2008, *24*, 1973-1978.
74. Stevens JB, Abdallah BY, Liu G, Horne SD, Bremer SW, Ye KJ, Huang JY, Kurkinen M, Ye CJ, Heng HH. Heterogeneity of cell death. *Cytogenet Genome Res*. 2013;*139*:164-73.
75. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol*. 2013;*5*:a008656.
76. Kaczmarek A, Vandenabeele P, Krysko DV. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. *Immunity*.2013;*38*:209-23.
77. Tekpli X, Holme JA, Sergent O, Lagadic-Gossmann D. Role for membrane remodeling in cell death: implication for health and disease. *Toxicology*. 2013;*304*:141-57.
78. Chaabane W, User SD, El-Gazzah M, Jaksik R, Sajjadi E, Rzeszowska-Wolny J, LosMJ. Autophagy, apoptosis, mitoptosis and necrosis: interdependence between those pathways and effects on cancer. *Arch Immunol Ther Exp (Warsz)*. 2013;*61*:43-58.
79. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 1983, *65*:55-63.
80. Draper, H.H.; Hadley, M. A review of recent studies on the metabolism of exogenous and endogenous malondialdehyde. *Xenobiotica* 1990, *20*: 901-910.
81. Chancerelle, Y.; Kergonou; J.F.. Immunologic relevance of malonic dialdehyde. *Ann Pharm Fr.* 1995;53241-250.
82. Cazzola R, Russo-Volpe S, Cervato G, Cestaro B. Biochemical assessments of oxidative stress, erythrocyte membrane fluidity and antioxidant status in professional soccer players and sedentary controls. *Eur J Clin Invest.* 2003;*33*:924-30.
83. Bonincontro, A; Di Ilio, V.; Pedata, O.; Risuleo G. . Dielectric properties of the plasma membrane of cultured murine fibroblasts treated with a nonterpenoid extract of Azadirachta indica seeds. *J. Membr. Biol.* 2007, *215*:75-79.
84. Cosimati R,Milardi GL,Bombelli C,Bonincontro A,Bordi F,Mancini G,Risuleo G. Interactions of DMPC and DMPC/gemini liposomes with the cell membrane investigated by electrorotation. *Biochim Biophys Acta.*2013;*1828*:352-6.
85. Milardi GL, Stringaro A, Colone M, Bonincontro A, Risuleo G. The Cell Membrane is the Main Target of Resveratrol as Shown by Interdisciplinary Biomolecular/Cellular and Biophysical Approaches. *J Membr Biol.* 2013. [Epub ahead of print] PubMed PMID: 24166779.
86. Berardi V, Aiello C, Bonincontro A, Risuleo G. Alterations of the plasma membrane caused by murine polyomavirus proliferation: an electrorotation study. *J Membr Biol.* 2009;*229*:19-25.
87. Andrabi SA, Dawson TM, Dawson VL. Mitochondrial and nuclear cross talk in cell death: parthanatos. *Ann N Y Acad Sci.* 2008;*1147*:233-41.
88. Li, J.; Yuan, J. Caspases in apoptosis and beyond *Oncogene* (2008) *27*, 6194–6206.
89. Van Blitterswijk W. J., Van Hoeven R. P. and Van Der Meer B. W., Lipid structural order parameters (reciprocal of fluidity) in biomembranes derived from steady-state fluorescence polarization measurements. *Biochim Biophys Acta.* 1981;*644*:323-32.
90. Van Blitterswijk W. J., in: Physiology of membrane fluidity, CRC Press, Taylor & Francis Group, London UK. Vol. II, 1984
91. Shinitzki M., in: Physiology of membrane fluidity, CRC Press, Taylor & Francis Group, London UK. Vol. I, 1984.

92. Russo L, Berardi V, Tardani F, La Mesa C, Risuleo G. Delivery of RNA and its intracellular translation into protein mediated by SDS-CTAB vesicles: potential use in nanobiotechnology. *Biomed Res Int.* 2013; *2013*:734596.