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Interaction enthalpy of lysozyme with vesicles

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Abstract

Negatively charged vesicles made of non-stoichiometric amounts of sodium dodecylsulfate and cetyltrimethylammonium bromide were characterised by physico-chemical methods. Interactions with lysozyme were examined by calorimetry. A remarkable heat effect is observed upon diluting the protein with the vesicular pseudo-solvent. The maximum ΔH_{bind} value occurs at complete vesicle charge neutralisation by the protein.

Keywords: Proteins; Vesicles; Surfactant mixtures; Interactions; Calorimetric studies; Interaction enthalpy; Thermodynamics

1. Introduction

Vesicle-based systems are appealing cell-mimetic models [1,2]. Much interest arises from the possibility of miming DNA interactions with cells [3]. Unfortunately, vesicles are metastable and efforts are devoted to get stable vesicles by use of synthetic lipids [4] or mixing surfactants and/or sterols [5]. Kaler and co-workers [6] and Khan and co-workers [7,8] demonstrated the possibility of forming stable vesicles by mixing oppositely charged surfactants in non-stoichiometric ratios. The resulting entities are thermodynamically stable [9], and their interactions with biopolymers can be determined in equilibrium conditions.

Vesicles formed by mixing anionic and cationic surfactants, termed "cat-anionic", are not cytotoxic [10]. They find use as transfection vectors, replace more conventional lipids, are electrically charged, and interact with proteins and DNA in a wide range of pH and ionic strengths. Electrostatic contributions to vesicle stability are significant and may be tuned by changing the ratios of the components. Charges facing outside the vesicles favour stabilisation, ensure significant interactions with charged biopolymers, and allow the formation of lipo-plexes, which are promising subjects for fundamental studies and transfection technologies.

Electron microscopies, fluorescence methods and spectroscopic investigations give information on biopolymer binding onto vesicles, on modifications of their size, of the electrical double layer and on the related energy contributions [11]. DSC has been widely used [12–14], but isothermal calorimetric studies (titration or batch) are limited to a few cases [15,16].

Thermodynamic analysis of the interactions between protein and cat-anionic vesicles is lacking. In this contribution, solution calorimetry, transmission electron microscopy, TEM, transport properties and surface tension were used to get self-consistent information on the interactions between cat-anionic vesicles and lysozyme, hereafter named LYSO. Such studies support the results inferred from electrophoretic mobility, light scattering and dielectric relaxation [17]. The physico-chemical properties of the bare vesicles are considered first. Thereafter, the interactions with proteins were investigated and some thermodynamic properties of the resulting lipo-plexes are reported. This contribution sheds light on some thermodynamic aspects of these systems and defines which forces control protein binding onto vesicles. Protein binding, insertion and adsorption onto cells and vesicles are important in biological systems, but not much is known of the specific events involved in protein surface adsorption at a molecular level and on the associated energy effects.

2. Experimental

2.1. Materials

Egg-white lysozyme, Sigma, was dialysed in 0.15m NaCl, recovered, dried, lyophilised, and kept over P_2O_5. Its purity was

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checked by ionic conductivity, density and viscosity in doubly distilled water. The results are in agreement with previous data [18,19].

Cetyltrimethylammonium bromide, CTAB, and sodium dodecylsulfate, SDS, were from Aldrich. The absence of minima in surface tension, γ, versus log surfactant molality plots close to the respective critical micellar concentrations (CMC), confirmed their purity [20].

Solutions of the anionic and cationic surfactants in micellar form were prepared individually and mixed together. For certain mole ratios the vesicles form rapidly; their dispersions are fluid, slightly turbid and colored. Vesicles are stable and no precipitate settles out after some months at room temperature. Particularly stable is the vesicular system [SDS]/[CTAB], mole ratio = 1.7, when the overall surfactant content is 6.0 mmol l^{-1} [21]. Protein–vesicle mixtures were prepared by dissolving the protein in the vesicular dispersions under stirring. The resulting dispersions were diluted with the vesicular pseudo-solvent and used within 1 day.

The pH of the mixtures, controlled by a 2000 Crison potentiometer, resulted to be 6.5 ± 0.2, and was independent on protein content. In such conditions LYSO has eight positive charges in excess [22]. Buffers were not used, to minimise the binding of interfering ions onto the protein or the vesicles, and to modulate their surface charge density and double layer thickness. This procedure is justified by the need to maximise the electrostatic interactions between the components.

2.2. Methods

2.2.1. Dilution enthalpy

Batch solution calorimetry at $25.00 \pm 0.01\,^{\circ}C$ was done with an LKB mod. 2107, as reported elsewhere [23]. Performance was checked by measuring the dilution heat of aqueous sucrose [24]. Integral enthalpies of dilution, ΔH_{dil} (J mol^{-1}) were expressed as

$$\Delta H_{dil} = \left[\frac{Q_{meas}}{n}\right] \quad (1)$$

where n is the moles of solute. The uncertainty on ΔH_{dil} values is ±1.0%. The ΔH_{dil} data were transformed according to

$$\Delta H_{dil} = \left(\frac{Q_{meas}}{n}\right) = (\Phi_{L,2,fin} - \Phi_{L,2,in}) \quad (2)$$

where $\Phi_{L,2,in}$ and $\Phi_{L,2,fin}$ are the relative apparent molal enthalpies of the protein before and after dilution with water or the vesicular pseudo-solvent, respectively. The $\Phi_{L,2}$ values were elaborated by a polynomial fit of the protein molality, m, and expressed as

$$\Phi_{L,2} = \sum_{i=1}^{i=3} A_i N m^{i/2} \quad (3)$$

where A_1 depends on the dielectric permittivity of the medium [25] and N indicates the number of ionised binding sites on the protein (eight in the pH conditions of this investigation). The other constants, A_2 and A_3, were calculated by

$$\left[\frac{\Delta H_{dil} - A_1(\sqrt{m_{fin}} - \sqrt{m_{in}})}{(\sqrt{m_{fin}} - \sqrt{m_{in}})^2}\right] = A_2 + A_3(\sqrt{m_{fin}} - \sqrt{m_{in}}) \quad (4)$$

Higher order terms in Eq. (4) were neglected, since their contribution is less than 1.0%, in such concentration regimes.

2.2.2. Surface activity

Measurements were performed by a Kruss K10T unit, equipped with a Du Noüy platinum ring, which was flamed, cleaned with 1.0m HCl and exhaustively washed with doubly distilled water. The temperature in the vessel was $25.0 \pm 0.1\,^{\circ}C$. The apparatus was calibrated before use with water and absolute ethanol. SDS micellar solutions were added with progressive amounts of aqueous CTAB, to get the vesicles. To minimise adsorption kinetics effects, the measurements were performed 10 min after addition of each CTAB aliquot. γ values in Fig. 1 are the average of five independent runs, and the resulting uncertainty was ± 0.2 dyn cm^{-1}.

2.2.3. TEM

Drops of the vesicular dispersions were adsorbed onto carbon-coated copper grids; the liquid in excess was removed. A drop of 1.0 wt% phospho-tungstic acid solution was stratified and the excess eliminated by filter paper. The samples were dried and observed by a ZEISS EM 900 electron microscope, at 80 kV [26] (Fig. 2).

2.2.4. Ionic conductivity

A Wayne-Kerr 6425 analyser was used. The measuring unit is located in an oil bath, at $25.000 \pm 0.003\,^{\circ}C$. As to vesicles formation is concerned, known amounts of CTAB solutions were added to micellar SDS, under stirring. χ values ($\mu S\ cm^{-1}$) were recorded 10 min after each addition. Significant changes in slope in χ versus added CTAB indicate vesicle formation.

Protein binding was inferred by adding the vesicular pseudo-solvent to concentrated water–protein–vesicle systems. The

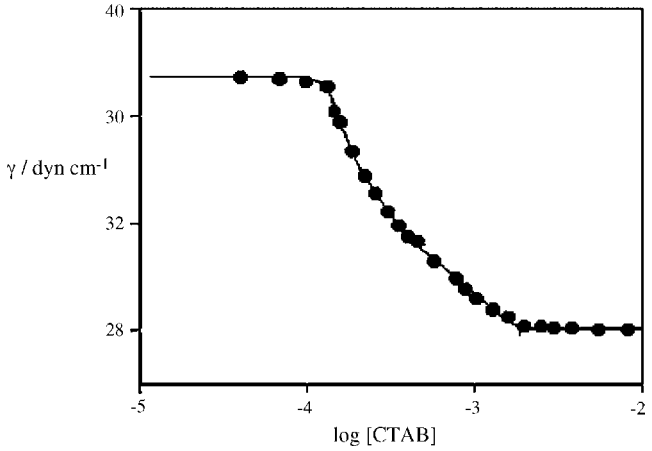

Fig. 1. The surface tension, γ (dyn cm^{-1}), of a 10.0 mmol kg^{-1} SDS solution, at 25.0 °C vs. the molality of added CTAB, in logarithmic scale. Vesicles are observed above the plateau region, in the lower left hand side of the figure.

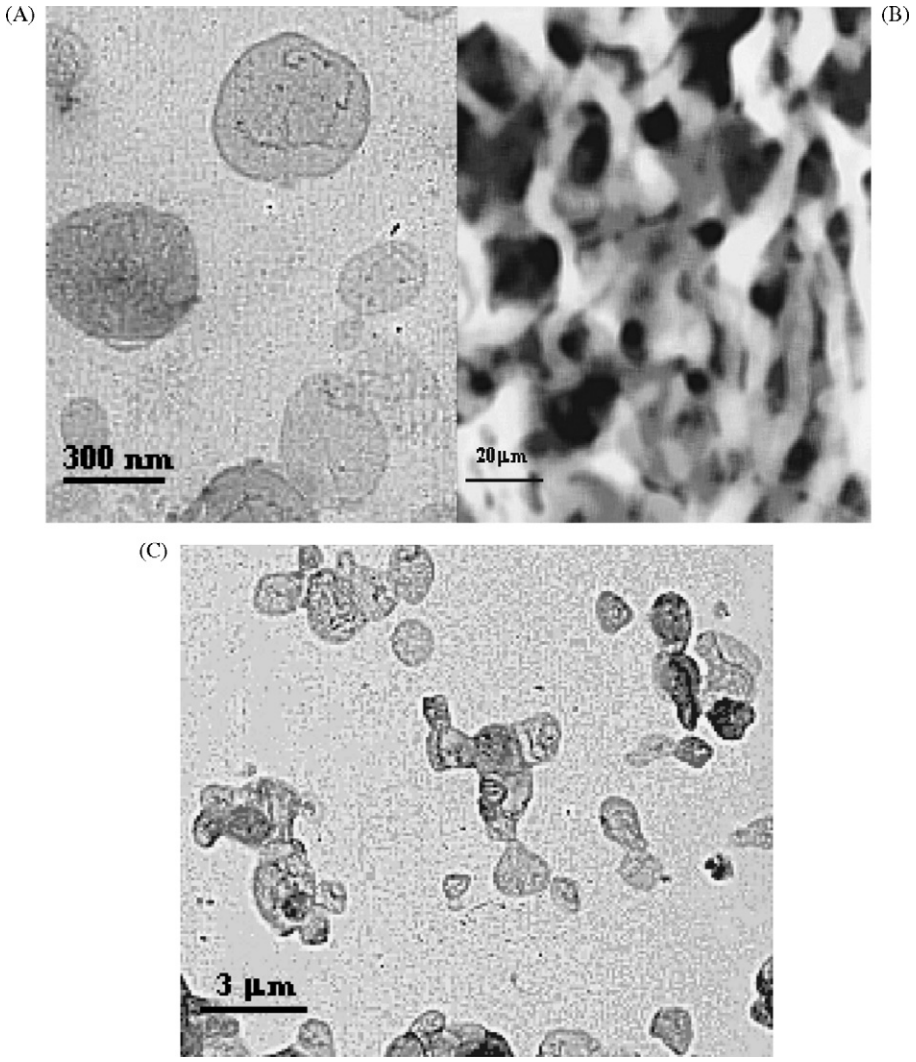

Fig. 2. (A) TEM image of (SDS/CTAB) vesicular dispersions. The overall surfactant content is 6.0 mmol kg^{-1} and the SDS–CTAB ratio (1.7/1.0). (B) TEM image of (SDS/CTAB) precipitates. The ratio between the two is 1.0/1.0 and the overall surfactant content 10.0 mmol kg^{-1}. (C) Image of SDS–CTAB vesicles, the same as in (A), upon addition of 0.14 mmol kg^{-1} of lysozyme. The particles size can be inferred by the bars in the bottom of figures.

absence of flocculation, or sedimentation was carefully controlled. No hysteresis in χ versus LYSO concentration plots was observed and the data uncertainty is lower than 0.5%.

2.2.5. Solution viscosity

An Ubbelohde capillary viscometer was located in a water bath at 25.00 ± 0.01 °C. Density measurements were made by an A. Paar DMA 60 unit at 25.00 ± 0.01 °C. Relative viscosity, η_{rel}, is the ratio $(t\rho/t^{\circ}\rho^{\circ})$, where t, t° and ρ, ρ° are the flow times and the densities of the mixtures and of the SDS solution, respectively. The precision of η_{rel} values in Fig. 3 is lower than ±0.5%. Vesicles onset is concomitant to changes in slope.

3. Results

3.1. Vesicle formation

Addition of CTAB to micellar SDS is associated with a significant decrease of surface tension values, followed by a plateau

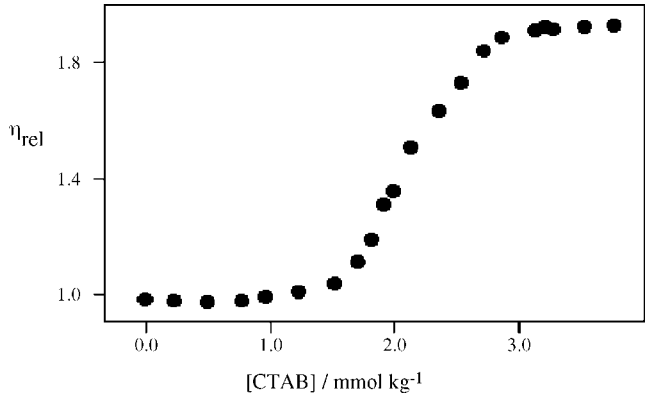

Fig. 3. The relative viscosity, η_{rel}, of a 10.0 mmol kg^{-1} SDS solution, at 25.00 °C, vs. the molality of added CTAB, mmol kg^{-1}. Vesicle onset is the plateau region in the top of the figure.

(Fig. 1). This behaviour is ascribed to significant changes in the area per molecule at the air–water interface and at the vesicle surface, due to efficient molecular packing of the components and matches the very low surface tension of lamellar dispersions compared to micellar solutions [27]. At still higher concentrations, precipitation of the poorly soluble (1–1) CTA–DS complexes occurs and measurements are not reliable.

Electron microscopy indicates the occurrence of unilamellar vesicles in the SDS–CTAB mixture, having sizes in the range 200–500 nm. Compared to other systems [28], the vesicles are quite mono-disperse in size, presumably because of their high charge density. The size distribution function is unimodal. Close to the phase separation limit, where non-soluble 1–1 (CTAB/SDS) complexes occur, crystals coexist with vesicles and other particles (Fig. 2).

Addition of lysozyme to vesicles results in the formation of lipo-plexes, having much larger sizes than the bare vesicles. Typical sizes may be some μm and the distribution function is broad. The quality of TEM pictures is much lower than in the corresponding vesicular systems (Fig. 2), but lipo-plexes are clearly detected.

Micelle–vesicle transitions are concomitant with modifications in the aggregate size and are reflected in the viscometric trends. When the aggregates retain their size and shape, the relative viscosity data increase with the volume fraction of the disperse phase, according to Einstein's equation [29]. In presence of size and shape transitions, conversely, sudden changes in slope occur [30]. Presumably, the transitions from micelles to vesicles result in a progressive increase of the particles size, passing through the formation of rods or other anisometric aggregates. This hypothesis agrees with the behaviour observed in the formation of lecithin-bile salt vesicles [31]. Accordingly, a steep viscosity increase (at low CTAB/SDS mole ratios) is followed by a smooth increment when vesicles dominate (Fig. 3).

In micellar systems the overall ionic mobility is due to the presence of molecularly disperse surfactants, free counter-ions and of micelles. The contribution due to micelles is by far the smallest and allows one to detect the amount of free counter-ions in such complex mixtures [32]. Turq and co-workers [33] showed that the conductometric behaviour can be rationalised in terms of concentrations of the single ionic species (including charged micelles), modulated by the respective diffusivities and relaxing contributions. Even more complex is the behaviour when CTAB forms mixed aggregates with SDS, releasing sodium and bromide ions. When vesicles are formed, no more (or moderate) counter-ion release occurs and the χ versus CTAB concentration plot becomes constant (Fig. 4). Addition of lysozyme to the vesicular dispersions, conversely, is concomitant to a noticeable increase in χ values, followed by a change in slope when vesicles are saturated by the protein (data not shown).

3.2. Protein binding

Many physico-chemical properties change when protein binding onto vesicles occurs. Experimental data give information on interaction energy, surface charge density and formation

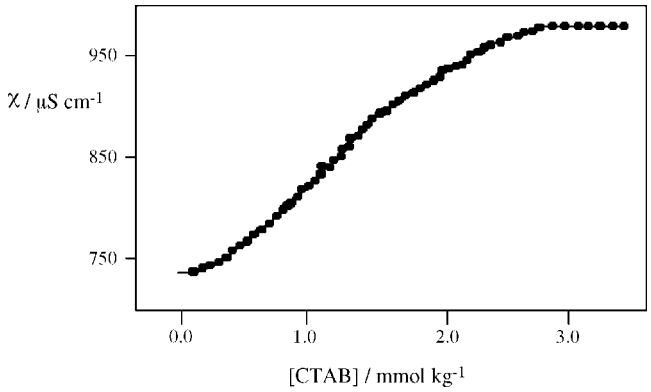

Fig. 4. The ionic conductivity, χ ($\mu S\,cm^{-1}$) vs. the molality of added CTAB, mmol kg^{-1}, to a 10.0 mmol kg^{-1} SDS solution, at 25.00 °C.

of lipo-plexes. The charge density of the vesicles ensures that the interactions with lysozyme involve, essentially, electrostatic contributions. To maximise double layer effects and counter-ion release from the vesicles no buffers were added. Electrolytes in excess, in fact, keep constant the Debye's screening length, reduce the vesicle charge density and the number of available binding sites.

The partial molal enthalpies of dilution for the protein, L_2, were obtained from $\Phi_{L,2}$ ones, according to

$$L_2 = \left(\frac{\partial(\Phi_L m^{1/2})}{\partial m^{1/2}} \right) \quad (5)$$

Data refer to the dilution of lysozyme in the vesicular solvent or in water. The negative partial molal enthalpies of dilution of lysozyme in pure water are reported in Fig. 5. As mentioned above, pH is practically constant (to within ±0.2 units). Therefore, calorimetric values do not contain de-protonation contributions due to lysozyme.

A sudden increase, followed by a steep discontinuity in L_2 values was observed by adding the protein to the vesicu-

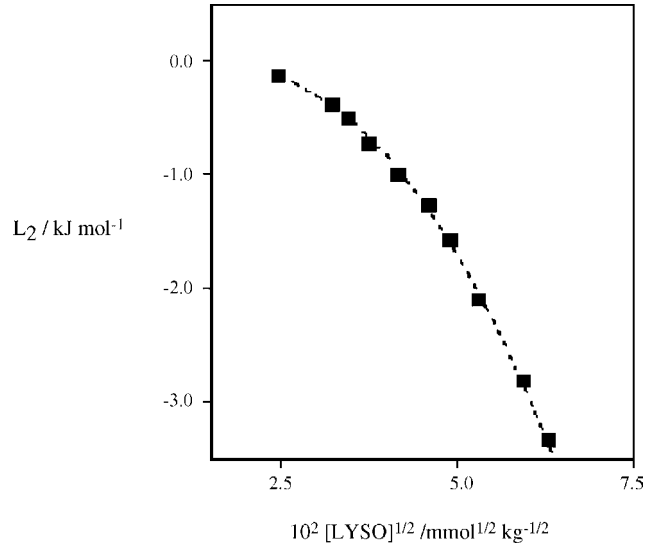

Fig. 5. The partial molal enthalpy of dilution of lysozyme, L_2 ($kJ\,mol^{-1}$), vs. the square root of lysozyme molality, 10^2 $mol^{1/2}\,kg^{-1/2}$, at 25.00 °C.

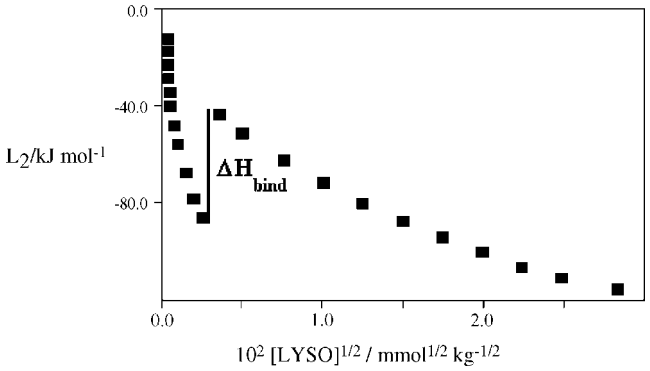

Fig. 6. The partial molal enthalpy of dilution of lysozyme, L_2 (kJ mol^{-1}), in 6.0 mmol kg^{-1} [SDS]/[CTAB], mole ratio = 1.7, vesicular pseudo-solvent vs. the square root of lysozyme molality, 10^2 mol$^{1/2}$ kg$^{-1/2}$, at 25.00 °C.

lar pseudo-solvent. At the neutralisation threshold the binding enthalpy of lysozyme, ΔH_{bind}, is -47.0 kJ mol^{-1} (Fig. 6). Such value implies the presence of large and negative enthalpic contributions, since the protein binding to vesicles is energetically favoured. The results also indicate that charge neutralisation and/or double layer interactions are relevant.

Above saturation, water, free lysozyme, lipo-plexes and counter-ions released from the vesicles coexist. In this concentration regime, the difference between L_2 values in water and in the pseudo-solvent corresponds to the transfer enthalpy of LYSO to a lipo-plexes containing solvent. Such difference is a regular function of protein content.

4. Discussion

In a first approximation, binding consists of uniform coating of the vesicle surface by small polyions. If all binding sites are equivalent, the process continues until all charges on the vesicles are neutralised. In the present work, no protein translocation across the bilayers is assumed, since globular proteins (such as lysozyme) prefer adsorption to insertion [34]. The protein is a spherical uniformly charged particle of 3–4 nm in diameter [35], and binding is rationalized in terms of hard spheres adsorption onto large vesicles surfaces. The charge density of the vesicles changes upon titration of the binding sites by the protein, with subsequent heat effects. The system investigated shows analogies with those where small polyions neutralise charged particles [36]. The number of titratable charges, uniformly distributed on the vesicles, is equal to the SDS concentration in excess.

Ionic conductivity supports the hypothesis of a stoichiometric vesicle titration. Changes in slope of χ occur at a concentration equivalent to that required to titrate SDS in excess. The change in slope occurs at nearly the same value at which the maximum heat effect is observed. Binding is thus surely responsible for the observed thermal effect.

The overall thermal effect is the sum of binding, counter-ion exchange [37] and desolvation [38,39]. Enthalpic terms due to changes in protein conformation may also occur [40], but the conformation of lysozyme (inferred by circular dichroism) does not change upon binding [21].

The protein adsorbs onto vesicles, up to full coverage, and reduces their charge density. This process favours the nucleation of protein-coated vesicles into lipo-plexes. It can be assumed that the residual charged groups on the protein join vesicles together. This hypothesis is due to the fact that charged groups onto lysozyme are uniformly distributed on the protein surface and, because of such geometrical constraints, only some of them neutralise a single vesicle.

According to experimental data, binding is concomitant to changes in the solution properties and formation of lipo-plexes. In energy terms, the enthalpy changes associated to such interactions are largely negative and ensure a not negligible contribution to the overall system stability. The resulting thermal effect is tentatively ascribed to the neutralisation of the electrical double layer around vesicles, with protein binding. Other contributions, such as de-hydration and counter-ion exchange, are also possible. It is difficult, perhaps, to quantify the relative weight of each of them, since most of the aforementioned contributions are interrelated.

References

[1] J.H. Fendler, Am. Chem. Soc. Symp. Ser. 342 (1987) 83.
[2] J.H. Fendler, Chem. Rev. 87 (1987) 877.
[3] S.M. Mel'nikov, R. Dias, Y.S. Mel'nikova, E.F. Marques, M.G. Miguel, B. Lindman, FEBS Lett. 453 (1999) 113.
[4] H. Eliyahu, N. Servel, A. Domb, Y. Barenholz, Gene Ther. 9 (2002) 850.
[5] S. Svenson, Curr. Opin. Colloid Interface Sci. 9 (2004) 201.
[6] L.L. Brasher, K.L. Herrington, E.W. Kaler, Langmuir 11 (1995) 4267.
[7] E.F. Marques, O. Regev, A. Khan, M. da, G. Miguel, B. Lindman, J. Phys. Chem. B 103 (1999) 8353.
[8] A. Khan, E.F. Marques, Curr. Opin. Colloid Interface Sci. 4 (2000) 402.
[9] E.F. Marques, Langmuir 16 (2000) 4798.
[10] S.K. Jung-Hua, J. Ming-Shiou, C. Chien-Hsiang, C. Hsuan-Wen, L. Cih-Ta, Colloids Surf. B Biointerfaces 41 (2005) 189.
[11] B. Lindman, S. Mel'nikov, Y. Mel'nikova, T. Nylaner, K. Eskilsson, M. Miguel, R. Dias, C. Leal, Prog. Colloid Polym. Sci. 120 (2002) 52.
[12] F.J. Aranda, J.A. Teruel, A. Ortiz, Biochim.Biophys.Acta—Biomembranes 1618 (2003) 51.
[13] N. Poklar, J. Fritz, P. Macek, G. Vesnaver, T.V. Chalikian, Biochemistry 38 (1999) 14999.
[14] A. Giehl, T. Lemm, O. Bartelsen, K. Sandhoff, A. Blume, Eur. J. Biochem. 261 (1999) 650.
[15] B. Nuscher, F. Kamp, T. Mehnert, S. Odoy, C. Haass, P.J. Kahle, K. Beyer, J. Biol. Chem. 279 (2004) 21966.
[16] (a) A.M. Rourke, Y. Cha, D. Collins, Biochemistry 36 (1996) 11913;
(b) D.A. Plager, G.L. Nelsestuen, Biochemistry 33 (1994) 7005;
(c) A. Ziegler, X.L. Blatter, A. Seelig, J. Seelig, Biochemistry 42 (2003) 9185.
[17] C. Letizia, P. Andreozzi, A. Scipioni, C. La Mesa, A. Bonincontro, E. Spigone, J. Phys. Chem. B 111 (2007) 898.
[18] A. Zielenciewicz, J. Therm. Anal. Calorim. 65 (2001) 467.
[19] K. Monkos, Biochim. Biophys. Acta—Prot. Struct. Mol. Enzymol. 1339 (1997) 304.
[20] K.J. Mysels, A.T. Florence, J. Colloid Interface Sci. 48 (1973) 410.
[21] C. Letizia, original graduate thesis, University of Rome "La Sapienza" (2005).
[22] B. Moernstam, K.G. Wahlund, B. Jönsson, Anal. Chem. 69 (1997) 5037.
[23] A.C. Palacios, M.L. Antonelli, C. La Mesa, Thermochim. Acta 418 (2004) 69.
[24] P. Monk, I. Wadso, Acta Chem. Scand. 22 (1968) 1942.
[25] P. Debye, L. Pauling, J. Am. Chem. Soc. 47 (1925) 2129.
[26] F. Iemma, U.G. Spizzirri, R. Muzzalupo, F. Puoci, S. Trombino, N. Picci, Colloid Polym. Sci. 283 (2004) 250.

[27] A.H. Alexopoulos, J.E. Puig, E.I. Franses, J. Colloid Interface Sci. 128 (1989) 26.
[28] R. Muzzalupo, S. Trombino, F. Iemma, F. Puoci, C. La Mesa, N. Picci, Colloids Surf. B Biointerfaces 46 (2005) 78.
[29] P.C. Hiemenz, Principles of Colloid and Surface Chemistry, second ed., M. Dekker, New York, 1986, p. 169.
[30] C. La Mesa, A. Bonincontro, B. Sesta, Colloid Polym. Sci. 271 (1993) 1165.
[31] P. Schurtenberger, N. Mazer, W. Kaenzig, J. Phys. Chem. 89 (1985) 1042.
[32] D. Stigter, J. Phys. Chem. 68 (1964) 3603.
[33] S. Duran-Vidal, P. Turq, O. Bernard, C. Treiner, J. Phys. Chem. B 101 (1713).
[34] C. Leggio, L. Galantini, E. Zaccarelli, N.V. Pavel, J. Phys. Chem. 109 (2005) 23857.
[35] J. Chamani, A.A. Moosavi-Movahedi, G.H. Hakimelahi, Thermochim. Acta 432 (2005) 106.
[36] S. Ulrich, A. Laguecir, S. Stoll, Macromolecules 38 (2005) 8939.
[37] M. Keller, A. Heinz, R.N. Lichtenthaler, Thermochim. Acta 229 (1993) 243.
[38] (a) Z. Liu, H.S. Chan, J. Mol. Biol. 349 (2005) 872;
(b) R. Brenk, S.W. Vetter, S.E. Boyce, D.B. Goodin, B.K. Shoichet, J. Mol. Biol. 357 (2006) 1449.
[39] N. Chouhury, B.M. Pettit, J. Phys. Chem. B 110 (2006) 8459.
[40] E. Grell, E. Schick, E. Lewitzki, Thermochim. Acta 380 (2001) 245.