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Dedicated to the memory of
Professor Ecaterina Ciorănescu-Nenitzescu (1909–2000)

ADSORPTION COMPLEXES OF COLLAGENOUS POLYPEPTIDE-IONIC SURFACTANT IN AQUEOUS MEDIUM

1. THE FORMATION OF MICELLAR STRUCTURE OF IONIC SURFACTANT ADSORBED ONTO COLLAGENOUS POLYPEPTIDE CHAIN

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The paper reports on the main findings issued from studying the formation of adsorbed micelle-like clusters of ionic surfactant (sodium dodecyl sulfate and hexadecyl trimethylammonium bromide) onto molecule of collagenous polypeptide (collagen hydrolyzates and calfskin collagen type I). Capillary viscometry, UV-circular dichroism, transmission electron microscopy and surfactant titration method were used to characterize the macromolecular component of the studied systems. To highlight the critical aggregation concentration (*cac*) and the critical saturation concentration (T_2) in the investigated polypeptide-surfactant systems, the steady-state fluorescence of pyrene (I_1/I_3 rule) and the surface tension measurements (the droplet volume method) were availed. The study revealed a significant decrease of the effective micelle concentration of surfactant (*cac*) in the presence of polypeptide irrespective of pH and a critical concentration T_2 depending on the system composition and pH.

INTRODUCTION

The importance of polypeptide-surfactant interactions, as a particular case of polymer-surfactant interactions, is contended by a lot of applications of these systems: food and pharmaceutical industry, analytical biochemistry.

In contradistinction to nonionic surfactants, which weakly interact with proteins without being able to denature them,[1] synthetic ionic surfactants exhibit a net higher interaction leading in some instances to the unfolding of native conformation of proteins. Thus, in the case of polypeptide chains having secondary structure, the binding isotherms (involving ionic surfactants) generally show four characteristic regions as concentration of the surface-active agent increases:[2] (a) specific binding, (b) non-cooperating binding, (c) cooperative binding, and (d) saturation. The specific binding is mostly electrostatically induced: the ionic heads of surfactants bind to the groups of the opposite-sign charge anchored to polypeptide chain. A pH variation of the environment will bring a variation in net charge of protein/polypeptide molecule, with consequences on the binding process. Commonly, if pH is low, the binding isotherm of an anionic surfactant entirely shifts to small concentrations of surfactant,[3] whereas the binding isotherm of a cationic surfactant shifts to larger concentrations of surface-active agent.[4] Depending on the primary structure of polypeptide, anionic surfactants will bind to the cationic sites (derived from residues of arginine - Arg, lysine - Lys and histidine – His), whilst cationic surfactants will bind to the anionic

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groups onto macromolecular chain (derived from residues of aspartic acid - Asp and glutamic acid - Glu). Nonetheless, the specific binding of an anionic surfactant seems to take place only if the length of its hydrocarbon tail reaches over threshold value.[3,5] Thus, it is found that sodium n-alchyl sulfates show a specific binding to lysozyme (pH 3.2) if their hydrocarbon tails contain 10 or 12 carbon atoms and do not exhibit this behavior at lower length of their tails (e.g. sodium octyl sulfate).[3] Similar results were found for binding of cationic surfactants such as n-alchyl trimethylammonium bromides to bovine serum albumin: the terms having 12 and 14 carbon atoms length undergo a specific binding, whereas decyl trimethylammonium bromide does not show this type of adsorption.[5] A non-cooperative binding process follows the saturation binding to specific sites onto protein/polypeptide which, in its turn, foregoes the stage of cooperative binding of surface-active agent encompassed within a narrow range of surfactant concentration. This is the region within which the adsorption protein/ polypeptide-surfactant complexes begin to take place, generating the micelle-like clusters of surfactants onto macromolecular chain. Some results suggest that a significant unfolding of native conformation of protein is produced during cooperative binding.[6] By means of UV-circular dichroism spectroscopy (UV-CD), an experimental technique very sensitive in observing the modifications of secondary structure of proteins, it could prove that the helical secondary structure of bovine serum albumin is damaged in proportion ranged between 66 and 50% with respect to its native conformation during binding of several anionic and cationic synthetic surfactants.[7] On the other hand, protein either shows minor variations of its secondary structure[8,9] or does not suffer any modification[10] in the case of sodium dodecyl sulfate (SDS) binding to lysozyme. At the same time, there are several works that indicate an increasing degree of helicity during SDS binding for some proteins, e.g., elastase, trypsin, and pepsin.[9-12] The saturation binding of ionic surfactants to protein/polypeptide is observed near critical micelle concentration (*cmc*) of surfactant and happens at a lower surfactant concentration for cationics than that for anionics.[13]

Binding studies of ionic surfactants to proteins in the systems consisting of opposite-charged components exhibit the possibility that the protein-surfactant complexes formed at low surfactant concentration to precipitate. Thus, a protein below its isoelectric point (IEP) carries a net-positive charge that may lead to protein precipitation during interaction with anionic surfactant (e.g., SDS), due to progressively diminishing of the net-positive charge of the protein-surfactant complex generated at low surfactant concentration.[14,15] Insoluble protein-surfactant complex will gradually become soluble at higher anionic surfactant concentration owing to the excess of negative charges carried by protein.

In the cooperative stage of binding, it is supposed that protein induces surfactant association in a process similar to regular micelles formation in surfactant solutions. The surfactant concentration at which this process starts is called critical association concentration[16] or critical aggregation concentration (*cac*).[17] The reach of the saturation binding generates saturated protein-surfactant complexes for which, at least in the case of SDS, four types of structure were proposed: (a) the rod-like particle model[18,19] proposed on the basis of viscosity measurements (the complex is a rigid rod with a radius of the cross section of 1.8 nm and a length proportional to the molecular weight of protein); (b) the flexible helix model[20] – a theoretical model which describes the protein-surfactant complex as a flexible cylindrical micelle formed by SDS molecules, on the surface of which hydrophilic segments of the protein molecules are adsorbed; (c) the necklace model[21] issued out of studies based on free-boundary electrophoresis of proteins (the model suggests an unfolding mechanism of the native conformation of a protein by the formation of SDS micelle-like clusters adsorbed onto polypeptide backbone); (d) the α-helix/random coil model[9] resulted on the basis of UV-CD data (the protein-surfactant complexes have a mixed structure that comprises α-helix/random coil arrangements with an unspecified SDS distribution within the complex). Among the aforementioned models, the necklace model seems to be supported by a lot of experimental findings: small-angle neutron scattering,[22-24] viscometry,[25] nuclear magnetic resonance,[26] nuclear magnetic resonance-electron spin resonance-fluorescence.[27] The necklace model describes a structure that resembles the structure of nonionic polymer-surfactant complexes,[28] or that of polyelectrolyte-surfactant complexes.[29]

The manifold applications of the collagen and its partially hydrolyzed products (e.g. gelatin, collagen hydrolyzates with different molecular weights) in the field of biomedicine (used in

surgical sutures, as haemostatic agents, for tissue replacement, in cardiovascular surgery, orthopedics, urology, neurology, ophthalmology), cosmetics (as ingredient in soaps, shampoos, face cream, body lotion) or food industry (by use of food-grade gelatin) have highly maintained the interest of scientists in studying such systems.[30] Practically, it is impossible to review the tremendous number of scientific paper and patents appeared since 1970s, when the producing and processing technology of novel collagen-based materials have been improved, by particularly obtaining collagen and its derivatives of medical grade.[31] Whether biomedical applications of collagen could be explained especially by its extremely low immunogenic effect and its use in food processing, as gelatin A or B, is based on its thickening and conditioning properties, the use of collagen or collagenous derivatives in cosmetics is debatable. The producers of a great number of cosmetics occurring in the market and containing collagen or collagenous derivatives as ingredients claim that these products show an anti-aging effect on skin. In fact, there are no credible studies to support such a presumption. The role of polypeptide component in this type of cosmetic formulations (soaps, shampoos), which have a significant amount of ionic surfactants, is presumably to minimize the secondary irritation effects on skin. Although the mechanism of dermal irritation by the ionic surface-active agents comprised in some cosmetic formulations is not fully understood, the surfactant in monomer (unassociated) form seems to be mainly responsible due to its high surface activity. This is the real reason for which nonionic or cationic polymers are contained in such formulations, in order to enhance the mildness of anionic surfactants towards skin. The process is due to reducing the effective critical micelle concentration of anionic surfactant by the formation of micelle-like aggregates of surfactant adsorbed onto polymer backbone at surfactant concentration much lower than that at which the regular micelles begin to form.[32]

Taking into account the above-mentioned aspects, in the present work the formation process of adsorbed collagenous polypeptide-ionic surfactant complexes at pHs different from IEP of macromolecular component is investigated, in both systems of ionic surfactant-polypeptide carrying a net charge with the same sign as surfactant and systems in which the two components have the opposite sign net charge. The primary aim of this study is to determine *cac* and surfactant concentration at the saturation binding in these systems, on the one hand, and to propose probable mechanisms on the formation of adsorbed complexes on the basis of the necklace and the rod-like particle models, on the other hand.

RESULTS AND DISCUSSION

The investigations regarding the formation of collagenous polypeptide-ionic surfactant complexes were achieved with two types of systems: (a) aqueous flexible collagenous polypeptide-ionic surfactant mixtures, in which polypeptide component was accounted for two collagen hydrolyzates and (b) aqueous collagen type I-ionic surfactant systems, in which the protein possesses a native triple helical structure. Vindication of the interactions in the studied systems is realized by both steady-state fluorescence (the method of fluorescence probes) and surface tension measurements (the droplet volume method).

1. Collagen hydrolyzate-ionic surfactant systems

Two collagen hydrolyzates, H1 and H2, were used as acidic and basic solutions. The acidic pH (2.5) was attained using glacial acetic acid and the basic pH by use of NaOH 0.1 N. SDS was utilized as anionic surfactant and hexadecyl trimethylammonium bromide (HTAB) as cationic surfactant. The polypeptide concentration was 0.5% (w/v) throughout the collagen hydrolyzate-ionic surfactant systems investigated.

The choice of two ionic surfactants took into account: highlighting of the onset of surfactant binding to the polypeptide backbone, which depends in a great extent on the length of the surfactant hydrocarbon tail[3,5] and avoiding of the significant variation of *cmc* with the system pH.

The ionic surfactant solutions at acidic and basic pHs were obtained in a similar way with the polypeptide-surfactant systems at the corresponding pHs. So, the acidic solutions of surfactant were prepared from their water solutions using then glacial acetic acid while the basic ones were obtained from the acidic systems by titration with NaOH 0.1 N. The slightly lower values of the *cmc*s for both surfactants at basic pH could be explained by a slight rise in the ionic strength of basic solutions as a result of NaOH titration (Fig. 1). Further explanations related to the plots significations are presented in detail below.

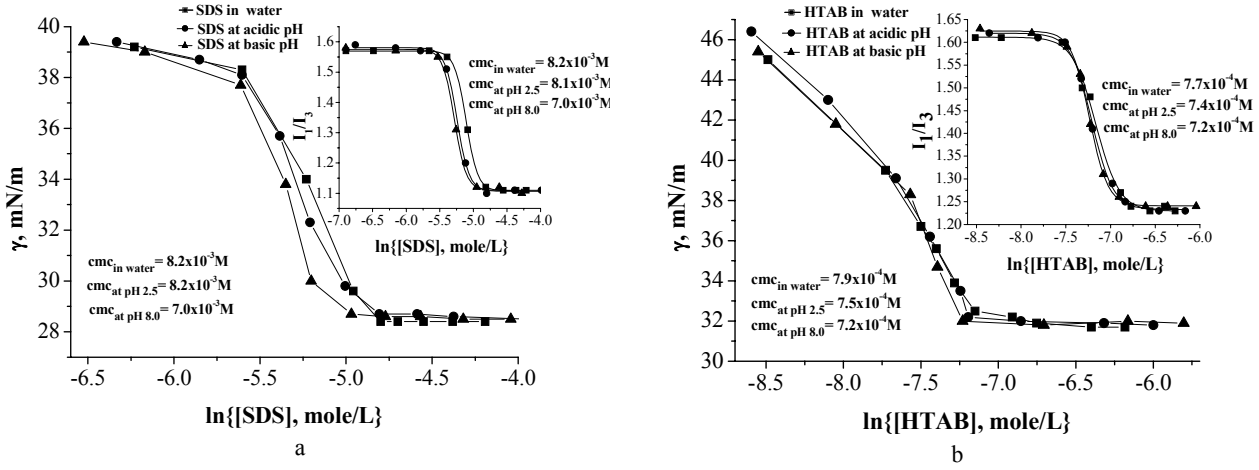


Fig. 1 – Surfactant concentration dependences of surface tension and I_1/I_3 ratio (inserted plots) for (a) SDS and (b) HTAB.

The characterization of the collagen hydrolyzates was realized by the determination of their molecular weight and isoelectric ranges.

The determination of molecular weight was made by capillary viscometry using the well-known relationship of Huggins:[33]

$$\frac{\eta_{sp}}{c} = [\eta] + k_H[\eta]^2 \cdot c \qquad (1)$$

where η_{sp} is the specific viscosity of solution, c – the solution concentration (in our experiments, in g/dL), k_H – the Huggins constant (dimensionless), and [η] – the intrinsic viscosity of solute (collagen hydrolyzate). From equation (1) it follows at once:

$$[\eta] = \lim_{c \to 0} \frac{\eta_{sp}}{c} \qquad (2)$$

From geometrical view point, [η] is given by the intercept of the linear dependence $\frac{\eta_{sp}}{c} = f(c)$ as resulted from equation (1). Knowing of the value of k_H for a certain system at a given temperature allows easily obtaining of [η] (by the single-point procedure), with a great advantage of avoiding of successive viscosity measurements for the same type of system but at different dilutions and, therewith, limitation of the sample consumption.[34,35] Because of the lack of k_H value for the two collagen-hydrolyzates, the method of successive dilutions was adopted and the plots are shown in figure 2.

The experimental conditions (37^0C and pH 4.0) have been chosen that the obtained intrinsic viscosities are valid in the Mark-Houwink-Sakurada (MHS) equation[36] in order to find out the molecular weights of H1 and H2:

$$[\eta] = K \cdot \left(\overline{M}_v\right)^a \qquad (3)$$

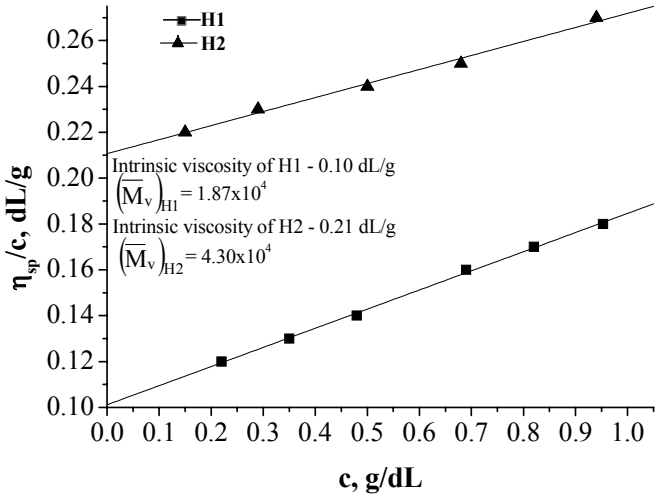


Fig. 2 – Reduced viscosity of the collagen hydrolyzates versus their concentration in aqueous solution at pH 4.0 and 37^0C.

In this equation, K is a constant with the same units of measurement as for [η], a is referred to as the exponent of MHS (dimensionless), and $\overline{M}_v$ is the viscosity average molecular weight of macromolecular compound in solution. The quantities K and a depend on the nature of polymer-solvent pair and temperature. The values of K (1.66×10^{-5} dL/g) and a (0.885) obtained by Pourardier and Venet for gelatin A at pH 4.75 and 37^0C[35-37] have been considered for H1 and H2. Therefore, the following values of $\overline{M}_v$ were found: 1.87×10^4 for H1 and 4.3×10^4 (in a.m.u.) for H2. The difference in their molecular weight explains the behavior of each H1 and H2 stock solutions (8%) during slow cooling: H1 solution preserved its liquid consistency down to the storage temperature (3-4^{0}C) while H2 stock solution underwent a gelification process at 17^0C.

The establishment of the isoelectric ranges of H1 and H2 was achieved by the method of ionic surfactant titration. In this respect, it is considered that at pHs lower than IEP any polypeptide carries a net-positive charge. The more acidic pH, the larger net charge of polypeptide will be. A similar layout, concerning a negatively-charged polypeptide, occurs at pHs higher than IEP. Although at IEP water solubility of H1 and H2 become lowest, the polypeptides still pretty soluble due to their rather small molecules. As a consequence, the variation of system turbidity on pH may be not large enough to precisely determine the isoelectric range of the collagen hydrolyzates. We considered, based on the situation described above, that the water solubility of polypeptide-ionic surfactant complexes (by monitoring turbidity) could significantly vary as a function of pH at a certain ratio of collagen hydrolyzate-ionic surfactant. Indeed, using aqueous collagen hydrolyzate-ionic surfactant systems with a constant polypeptide concentration (1 g/dL) and the same polypeptide/surfactant ratio (10/1 by weight) at various pHs, it is found that:

(a) in the case of SDS, the highest value of turbidity is attained at pH 3.0-3.5 and the lowest turbidity at pHs above 4.3-4.5;

(b) using HTAB, the highest turbidity is obtained at pH 8.0, thereafter it follows a decrease in turbidity down to its lowest value attained at pHs below 5.2-5.3 (Fig. 3).

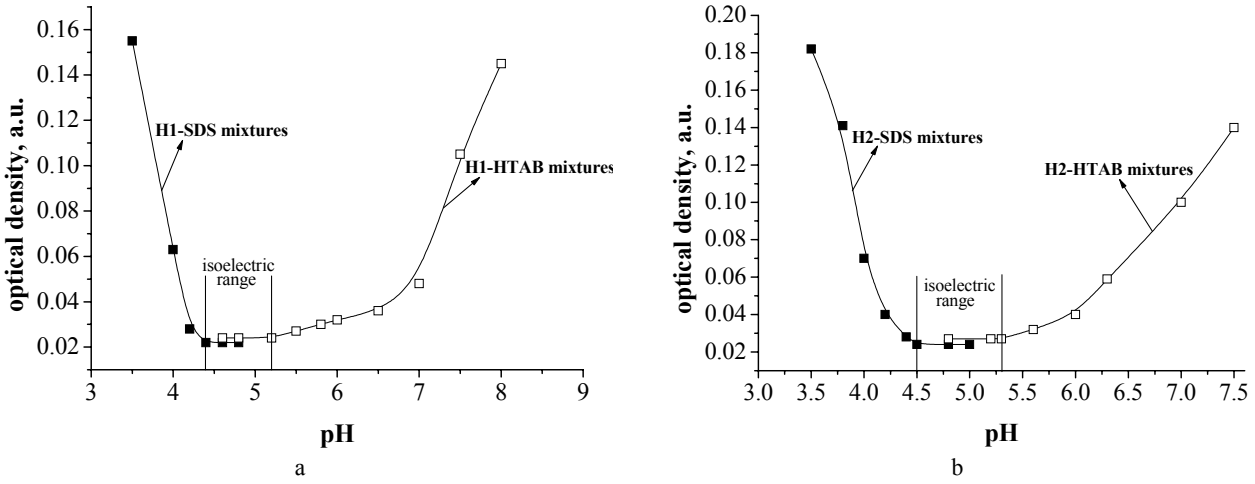


Fig. 3 – Variation of system turbidity as a function of pH for (a) H1 and (b) H2 in the surfactant titration experiments (at 23^0C).

The domains of the lowest turbidity values exhibited by the turbidity-pH dependences have been considered as the isoelectric ranges of the two hydrolyzates: 4.4-5.2 for H1 and 4.5-5.3 for H2 (Fig. 3).

1.1. Collagenous polypeptide-SDS systems. In order to highlight the interaction between macromolecular compound and ionic surface-active agent throughout the systems studied, both the steady-state fluorescence, using pyrene (Py) as fluorescence hydrophobic probe, and surface tension measurements (droplet volume method) were utilized.

The use of Py as fluorescence hydrophobic probe is based on so-called I_1/I_3 rule of Py.[38] According to that, the great sensitivity of I_1/I_3 ratio to the solvent micropolarity is a very useful tool in the correlation of values of I_1/I_3 ratio with different polarities of a lot of solvents.[39] In this respect, a so-called Py-scale was defined[40] by which solvent

polarities may be assessed. Thus, the lower polarity of a solvent the lower value of I_1/I_3 ratio and vice-versa. Extending of the application of I_1/I_3 rule to surfactant solutions and polymer-surfactant systems allowed determination of *cmc* and *cac*.[40-42] For instance, during the micelle self-association of surfactant, I_1/I_3 value steeply decreases which proves that Py is solubilized preferentially into the hydrophobic microdomains generated by micellization.[40] At the same time, a low value of I_1/I_3 ratio in a polymer solution, in comparison with that obtained in the solvent, indicates the formation of hydrophobic regions by either conformational changes of macromolecules or their intermolecular association.[43]

The concentration of Py for all investigated systems was cca $4x10^{-7}$ M to avoid producing the excimer species. The use of I_1/I_3 rule to assess both *cmc*s of surfactants and *cac*s in polypeptide/collagen-surfactant systems is based on the investigations achieved by Zana et al.[44,45] concerning *cmc* determination. Shortly, according to these studies, there are two approaches for *cmc* determination:

(a) by considering *cmc* as the abscissa of the crossing point between the extrapolation of the linear descending region I_1/I_3 = f(surfactant concentration) with the prolongation of the almost horizontally linear dependence located at high concentration of surfactant or

(b) expressing the *cmc* as the abscissa of the inflexion point of the I_1/I_3 - surfactant concentration dependence in the region of rapid decrease of I_1/I_3 as surfactant concentration increases.

The first route is designated to determination of the higher values of *cmc* (10^{-3}-10^{-2} M, depending on the surfactant) and the second is recommended for the lower *cmc*s (below 10^{-3} M). The last way is justified by the fact that Py is completely solubilized into the surfactant micelles having low *cmc* only after the volume of hydrophobic pseudophase became large enough.[45]

In the studied systems, taking into account the correlation between the fluorescence and surface tension results, the first route was used to find out *cmc* of SDS and the second route was applied to determine *cmc* of HTAB and *cac*s for all polypeptide/collagen-ionic surfactant systems.

As it has been shown elsewhere,[46] the abscissa of the inflection point mentioned above may be found by fitting the experimental data using a Boltzmann sigmoid function.

The choice of the droplet volume method for the surface tension measurements reckoned upon the following arguments: a low sample consumption, a measurement accuracy of ±0.1 mN/m and the possibility of drop growing under almost quasistatic conditions and saturated solvent atmosphere. Surface tension measurements enable detection of *cmc*s of surfactants, on the one hand, and *cac* and, possibly, the surfactant concentration at which the saturation binding of surfactant to polypeptide has been attained, on the other hand. The critical saturation concentration, symbolized by T_2, as Jones proposed for poly(ethylene oxide)-SDS system,[47] represents the surfactant concentration at which the surface tension in polypeptide-surfactant mixture is equal to the surface tension in surfactant solution within the micelle-forming region.

At pH 2.5 (below IEP of H1 and H2), the interaction between polypeptide and SDS shows the onset of complexation at a surfactant concentration much lower than *cmc* of SDS ($8.3x10^{-3}$ M).

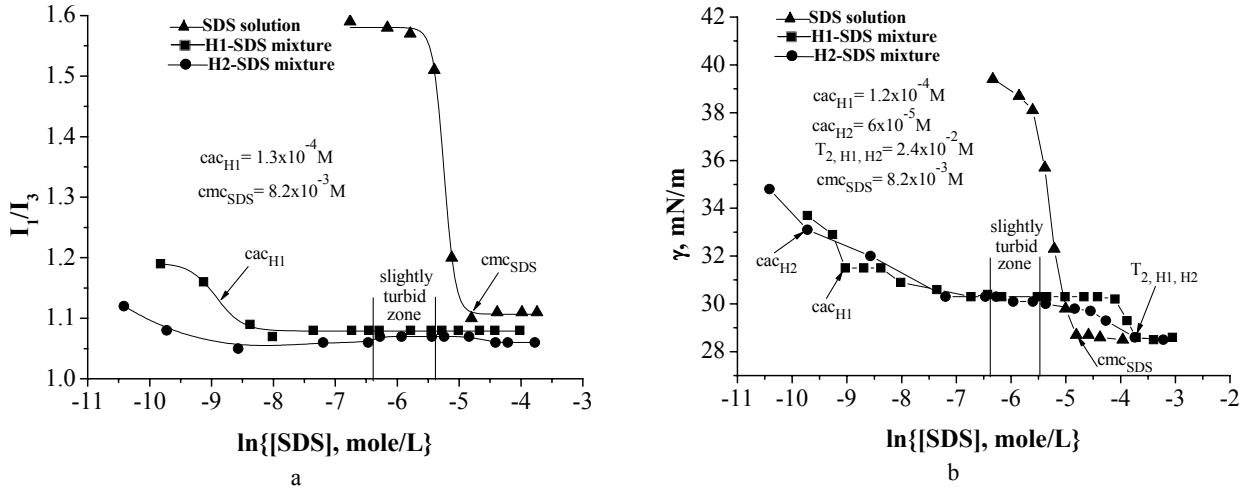


Fig. 4 – Dependences of (a) I_1/I_3 ratio and (b) surface tension on the total SDS concentration for the surfactant solution and the collagen hydrolyzate-SDS systems at pH 2.5 and 25^0C.

For H1, *cac* is $1.3x10^{-4}$ M – from fluorescence and $1.2x10^{-4}$ M – from surface tension measurements whereas for H2 there is no possibility to determine *cac* by means of fluorescence but the surface tension data indicate presumably a *cac* of about $6x10^{-5}$ M. Even if *cac*s found for H1 and H2 are pretty different, the critical concentration T_2 is the same for both hydrolyzates – $2.4x10^{-2}$ M. As it is shown in figure 4, the systems possess a slightly higher turbidity in the SDS concentration range $1.7\text{-}4.6x10^{-3}$ M, which denotes the formation of some low-soluble H1/H2-SDS complexes. The occurrence of *cac* values much lower than *cmc* of SDS proves the major importance of the electrostatic factor in inducing the polypeptide-surfactant interaction. At the same time, the random coil conformation (it may practically expect that the polypeptide chain has not any secondary structure) of H2 (having $\overline{M}_v$ higher than that of H1) generates by its net-positive charge a mean electrostatic potential per molecule that can induce the onset of the anionic surfactant aggregation at *cac* ≈ $6x10^{-5}$ M, much lower than in the case of H1 (*cac* $1.2\text{-}1.3x10^{-4}$ M). The micropolarity sensed by Py in its immediate vicinity shows that even if the micelle-like clusters of SDS formed at *cac* have a core polarity pretty similar to that of the regular micelles of SDS (Fig. 4a), the existing polypeptide-surfactant complexes engender hydrophobic microdomains having a polarity much lower than that of the SDS micelles as surfactant concentration increases above *cac*. This could be an indication of a low occurrence of water molecules in hydrophobic pseudophase generated during the complexes formation compared with the regular SDS micelles.

At pH 8.0, SDS adsorption onto polypeptide backbone is clearly highlighted by both fluorescence (Fig. 5a) and surface tension measurements (Fig. 5b).

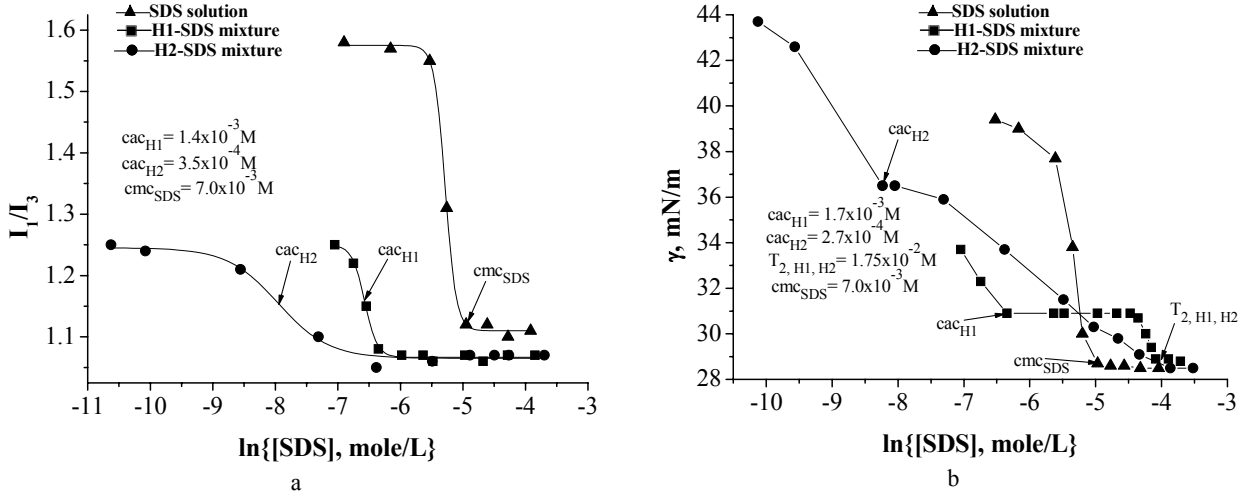


Fig. 5 – Variation of (a) I_1/I_3 ratio and (b) surface tension as a function of the total SDS concentration for the surfactant solution and the collagen hydrolyzate-SDS systems at pH 8.0 and 25^0C.

The same tendency was observed, but much more pronounced than at acidic pH: H2 shows a much lower *cac* ($3.5x10^{-4}$ M – fluorescence, $2.7x10^{-4}$ M – surface tension) than that for H1($1.4x10^{-3}$ M – fluorescence, $1.7x10^{-3}$ M – surface tension). Besides, the saturation adsorption is the same for both hydrolyzates ($T_2 \approx 1.8x10^{-2}$ M) but lower than that observed at acidic pH. The formation of polypeptide-SDS complexes in basic medium seems to be in contradiction with the role of the electrostatic influences played in the initiation and the development of interactions at low and very low concentration of surfactant. In other words, H1 an H2, at pH 8.0, will carry a net-negative charge which is practically unfavorable to obtain the adsorption complexes with an anionic surfactant such as SDS. Despite this fact, the real situation is more complex. As said above, depending on pH, the net-negative charges of a collagenous polypeptide derives from the carboxyl groups of Asp and Glu residues while the net-positive charges – from the amino groups belonging to Arg and Lys residues and the imidazole nitrogen of His. For simplicity, considering the mean values of the acidic exponents for –COOH/-COO^- (pK_a 2.04) and –NH_3^+/-NH_2 (pK_a 9),[48] a diagram of the amount of the charged groups versus pH can be plotted on the basis of the Henderson-Hasselbalch

equation (Fig. 6). It is easy to observe that all carboxyl groups are ionized to carboxylate and almost 91% of the amino groups are in the ammonium form at pH 8.0. Therefore, the existence of binding sites with net-positive charge onto polypeptide chain is, indeed, the favorable electrostatic factor in the earliest stages to promote SDS adsorption at concentration much lower than *cmc*. The formation of SDS clusters at *cac* by the significant contribution of the hydrophobic effect leads, as for acidic pH, to a hydrophobic pseudophase having a micropolarity (sensed by Py) similar to that for the regular micelles of the anionic surfactant. As SDS concentration increases, the hydrophobic character of the collagen hydrolyzates-SDS complexes become clearly more pronounced with respect to SDS micelles (Fig. 5a).

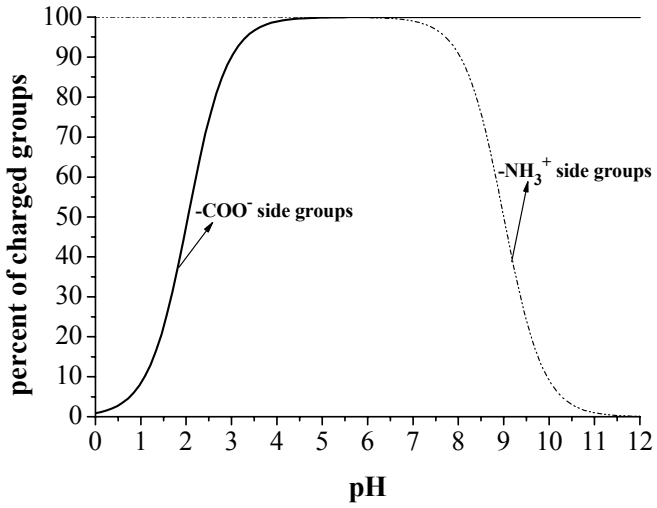


Fig. 6 – Relative amount of the charged side groups – COO^- (Asp, Glu) and $–NH_3^+$ (Arg, Lys) versus pH in a polypeptide.

1.2. Collagenous polypeptide-HTAB systems. At acidic pH (2.5), the interaction between the collagen hydrolyzates and the cationic surfactant might exhibit similar characteristics as for the collagen hydrolyzate-SDS mixtures at basic pH. In fact, the amino groups of the polypeptides are entirely protonated and the carboxyl groups are in carboxylate form in a proportion of cca 74% (Fig. 6), the latters being the specific binding sites of HTAB onto polypeptide chain. Both fluorescence and surface tension measurements show the beginning of surfactant association induced by the collagen hydrolyzates at HTAB concentrations of about one order of magnitude lower than its *cmc*: $8.3x10^{-5}$ M – from fluorescence and $7.3x10^{-5}$ M – from surface tension for H1; $5.8x10^{-5}$ M – from fluorescence and $5.5x10^{-5}$ M – from surface tension for H2 (Fig. 7).

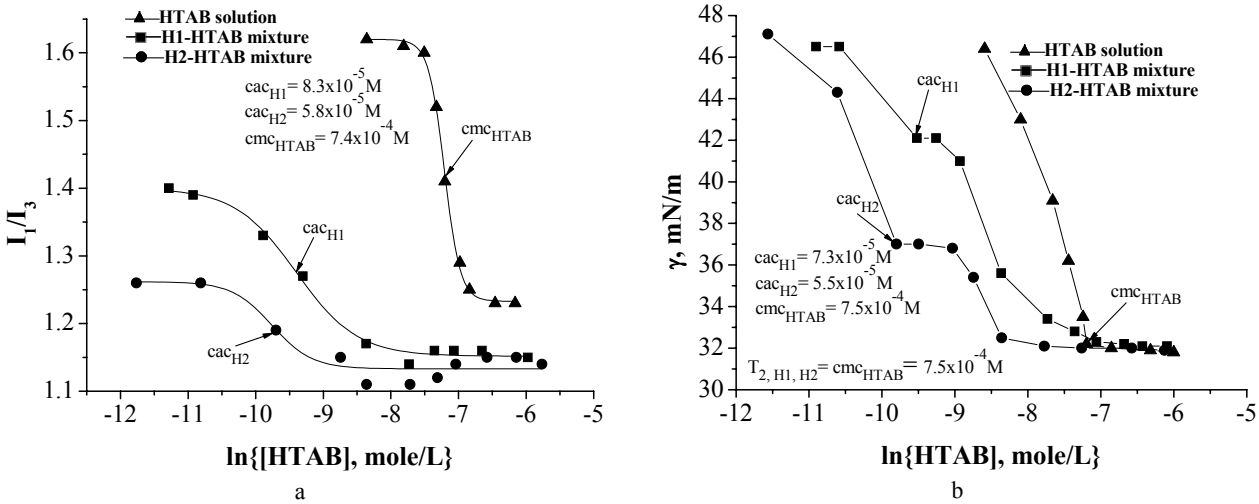


Fig. 7 – HTAB concentration dependences of (a) I_1/I_3 ratio and (b) surface tension for the cationic surfactant solution and the collagen hydrolyzate-HTAB systems at pH 2.5 and 25^0C.

It is interesting that the critical concentration T_2 for both hydrolyzates coincides with the *cmc* of HTAB (Fig. 7b). This finding could suggest the lack of HTAB adsorption onto the collagen hydrolyzates, which seems to be in disagreement with the florescence data. As shown in figure 7a, at HTAB concentration above the *cac*s for H1 and H2, the values of I_1/I_3 ratio diminish till they attain a plateau located much below that of micelle formation region in surfactant solution. This depicted situation is equivalent to the probable occurrence of two types of hydrophobic regions at surfactant concentrations above *cmc*: a pseudophase represented by the inside region of the regular micelles of the surfactant and a pseudophase representing the inside part of the associated structures of HTAB complexed with the polymeric component characterized by a micropolarity much lower than that of the core domain of the regular micelles of the cationic surfactant. Whereas the micropolarity resulted from fluorescence measurements is a weighted mean of the micropolarities sensed by Py solubilized in the interior of the two types of pseudophases, it is expected that the preponderant hydrophobic pseudophase is that generated during the formation of the collagen hydrolyzate-HTAB complexes. Thus, the correlation between the fluorescence results and those of surface tension leads to the supposition according to which the saturation adsorption of HTAB onto H1 and H2 could be significant but too difficult to assess it by surface tension measurements, probably due to a high surface activity of the H1/H2-HTAB complexes.

The investigation of the collagen hydrolyzate-HTAB systems at basic pH (8.0) reveals some special findings: (a) the net-negative charge of the polypeptides represents a favorable factor which induces the formation of the collagen hydrolyzates-HTAB complexes at low surfactant concentration; (b) regarding the assessment of *cac*, Py fluorescence indicates o good agreement with the surface tension data only for H1 (3.2×10^{-5} M – fluorescence, 3.5×10^{-5} M – surface tension) while, in the case of H2, it is not possible to fit satisfactory the fluorescence data versus HTAB concentration (to obtain *cac*) and the surface tension-surfactant concentration dependence shows a probable value of 2.5×10^{-5} M (Fig. 8); (c) the hydrophobic character of the inside part of HTAB clusters formed onto H1 chain is high in comparison with that of the regular micelles of HTAB (Fig. 8a), unlike the previous cases where the fluorescence measurements revealed the same micropolarity of polypeptide-surfactant aggregations at *cac* as for that of ordinary micelles of surfactant; (d) the surface tension data exhibits almost the same value of T_2 for both H1 and H2 and equal with *cmc* of HTAB – 7.2×10^{-4} M (Fig. 8b), as at acidic pH; (e) the disagreement between the fluorescence and surface tension data requires additional investigations in the future.

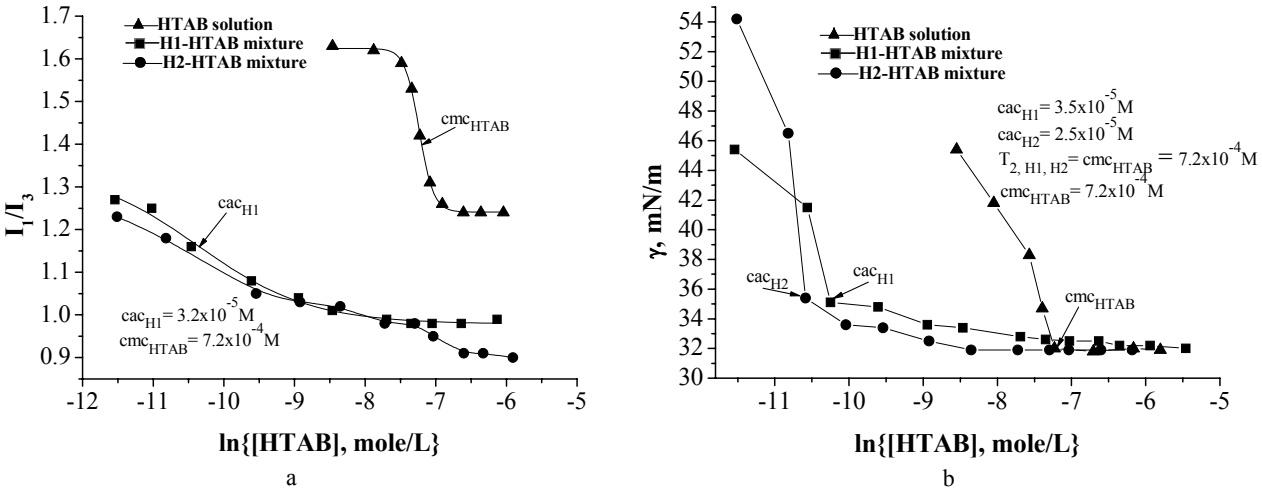


Fig. 8 – HTAB concentration dependences of (a) I_1/I_3 ratio and (b) surface tension for the surfactant solution and the collagen hydrolyzate-HTAB systems at pH 8.0 and 25^0C.

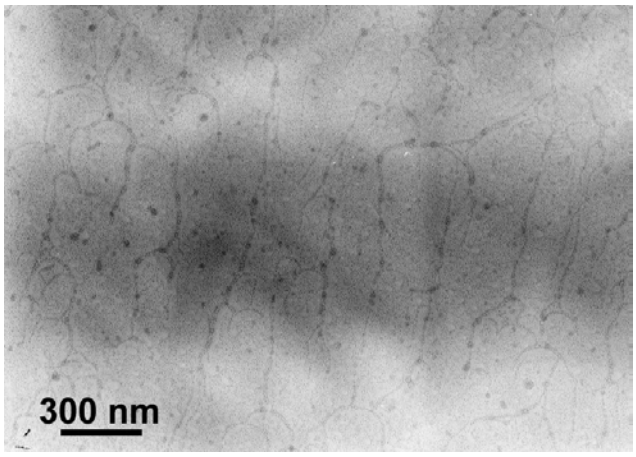
2. Collagen-ionic surfactant systems

Throughout the aqueous protein-surfactant systems, the concentration of the calfskin collagen type I of 0.05% (w/v) was chosen to obtain very low turbid dispersions of collagen suited to be investigated by Py fluorescence.

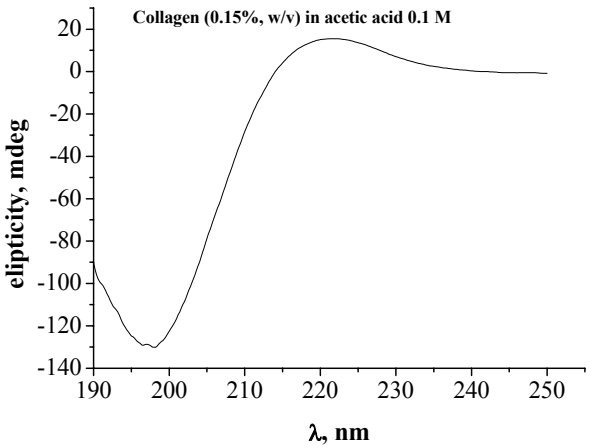
The characterization of the collagen type I referred the evaluation of its native secondary structure (triple helix) and the establishment of its isoelectric range and molecular weight as well.

The native structure of the collagen mainly implies an uninterruptible triple superhelical arrangement with about 300 nm in length and a diameter of 1.5 nm.[49] The molecular and conformational integrity were investigated by electron microscopy and UV-CD.

In figure 9a, the collagen molecules can be observed as hair-shaped structures of 250-280 nm in length. A low degree of supramolecular association and branching can also be seen..



a



b

Fig. 9 – Visualization of the molecular integrity and assessment of the native secondary structure of the collagen by (a) TEM and (b) UV-CD.

The triple helical secondary structure possesses an optically active conformational folding which exhibits some particular characteristics in UV-CD spectrum: a pronounced negative minimum located at 190-200 nm, a weak positive maximum at cca 220 nm and a crossover point at about 212 nm.[50-52] An important quantity which indicates the helicity degree of the collagen secondary structure is the absolute value of the ratio between the intensity of the positive peak and that of the negative peak in UV-CD spectrum of collagen (Rpn).[53,54] Rpn is 0.12 for the collagen with a native secondary structure.[55]

The UV-CD spectrum of the collagen used in this study is presented in figure 9b. The characteristic elements come from the spectrum (the positive peak – 222 nm, the negative peak – 198 nm, the crossover point – 214 nm, Rpn – 0.12) are in an excellent accordance with the existing literature data regarding the native structure of collagen.

The determination of molecular weight was performed, as for H1 and H2, by capillary viscometry (Fig. 10). For the collagen utilized in the measurements, an intrinsic viscosity of 12.26 dL/g is hereby obtained under experimental conditions (collagen solutions in acetic acid 0.15 M, 24.8^{0}C) for which the MHS equation constants are known: K = 1.23x10^{-9} dL/g and a = 1.80.[56,57] Considering these data, $\overline{M}_v$ was found to be 3.6x10^5 a.m.u.

The determination of the isoelectric range of the collagen was achieved by the visual observation of the collagen systems non-homogeneity (collagen concentration – 0.1% w/v) at different pHs. In this way, an isoelectric range 4.6-6.0 was obtained.

2.1. Collagen-SDS systems. At pH 2.5 (below the isoelectric domain of the collagen), the collagen-SDS interaction strongly favored by the electrostatic factor (the positive electrostatic potential per collagen molecule is much higher than that of H1 or H2 at the same pH) leads to insoluble complexes occurring a massive separation of collagen from solution at SDS concentration ranging from 2.3x10^{-4} M to 2x10^{-3} M (Fig. 11). At higher SDS concentrations, after the collagen solubilization, the environment micropolarity sensed by Py (preferentially localized within the hydrophobic microdomains of the collagen-SDS aggregates and SDS micelles) is a little lower than that measured only in the ordinary SDS micelles at the same pH (2.5).

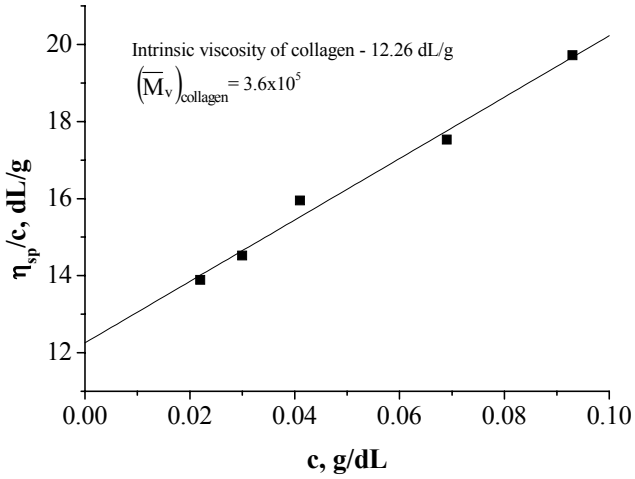


Fig. 10 – Concentration dependence of the reduced viscosity of the collagen in acetic solution (acetic acid 0.15 M) at 24.8^{0}C.

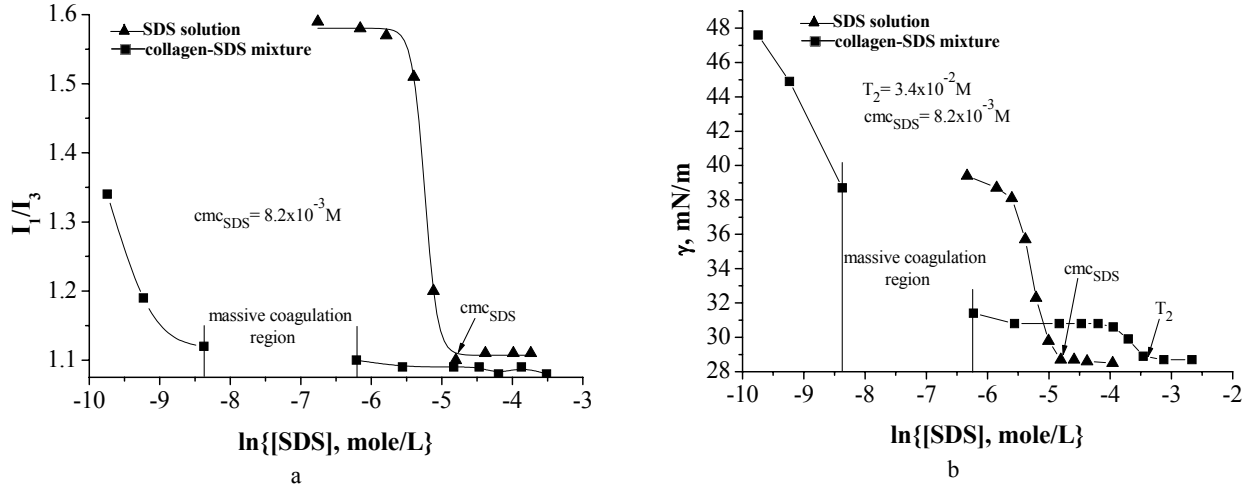


Fig. 11 – Variation of (a) I_1/I_3 ratio and (b) surface tension on the total SDS concentration for the anionic surfactant solution and the collagen-SDS system at pH 2.5 and 25^{0}C.

As seen in figure 11, neither by the fluorescence nor by the surface tension method can be observed *cac* in the collagen-SDS mixture due to the strong protein-SDS interaction occurred at the earliest stages of surfactant binding (the onset of SDS aggregation onto collagen probably takes place at very low, undetectable, surfactant concentration). The surface tension measurements in return indicate a critical concentration T_2 of 3.4x10^{-2} M (Fig. 11b).

At basic pH (8.0) the collagen-SDS system remains homogenous (monophasic) over the entire range of SDS concentrations. As a result of the fact that the majority of the amine groups are protonated at pH 8.0 (Fig. 6), SDS association onto the positive binding sites of protein begins at surfactant concentrations (1.4x10^{-3} M – from fluorescence, 1.3x10^{-3} M – from surface tension) lower than its *cmc* (Fig. 12).

It is important to retain that the micropolarity of the novel hydrophobic collagen-SDS pseudophase, which might be the favorite place for the Py solubilization at *cac*, is significantly higher than that of the regular micelles of SDS (Fig. 12a). This could be due to the one or both of the following aspects: SDS clusters adsorbed onto collagen helix contain water in a larger extent than the anionic surfactant micelles or the hydrophobic pseudophase appeared by SDS association at *cac* is small enough (the number of surfactant hemimicelles adsorbed onto the collagen molecule is rather small due to the electrostatic repulsions between hemimicelles) so that the overall micropolarity is a mean result of Py partitioning between the aqueous phase and the new-born hydrophobic pseudophase.

The situation depicted above is wholly different from that met in the collagen hydrolyzate-SDS systems, where the micropolarity exhibited by Py at *cac* is pretty the same as for that of SDS micelles (Fig. 4a and 5a). The occurrence of the significant saturation adsorption of SDS is clearly

observed by means of the surface tension measurements: the critical concentration T_2 is $2.1x10^{-2}$ M, lower than T_2 obtained at acidic pH (Fig. 12b).

2.2. Collagen-HTAB systems. The particularities of the collagen-HTAB interaction highlight several similitudes and contradistinctions with respect to the analogous collagen hydrolyzate-HTAB mixtures.

At acidic pH (2.5), the carboxyl groups, as carboxylate groups in preponderant proportion (Fig. 6), induce HTAB aggregation onto the collagen molecule at pretty different *cac*s as they were obtained from the measurements: $1.5x10^{-4}$ M – from fluorescence, $2.1x10^{-4}$ M – from surface tension (Fig. 13). However, these values are much higher than those for H1 and H2 (Fig. 7 and 8).

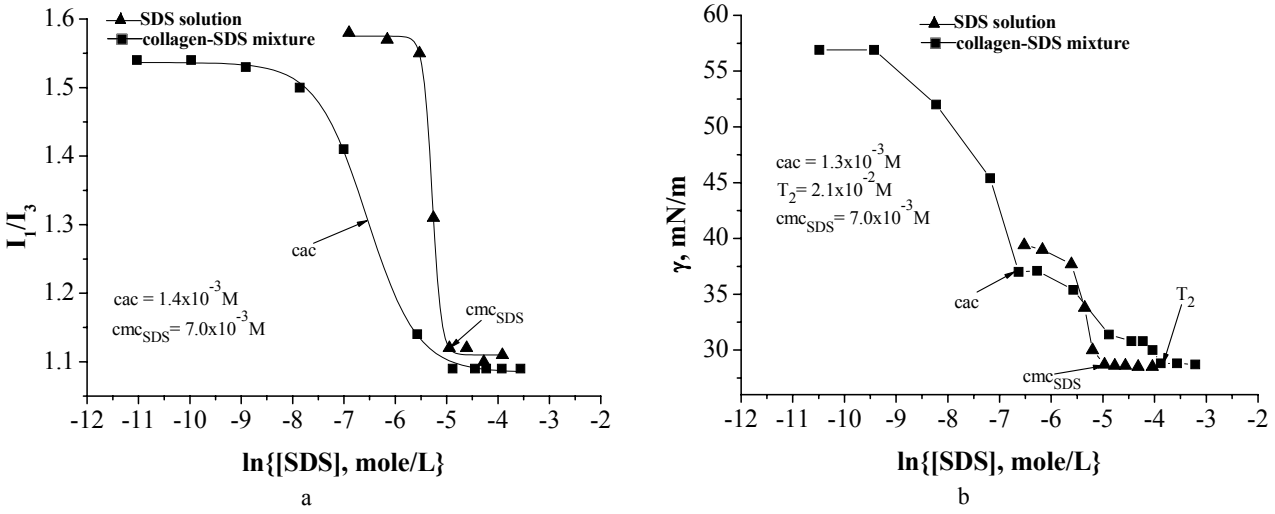


Fig. 12 – Variation of (a) I_1/I_3 ratio and (b) surface tension on the total SDS concentration for the anionic surfactant solution and the collagen-SDS system at pH 8.0 and 25^0C.

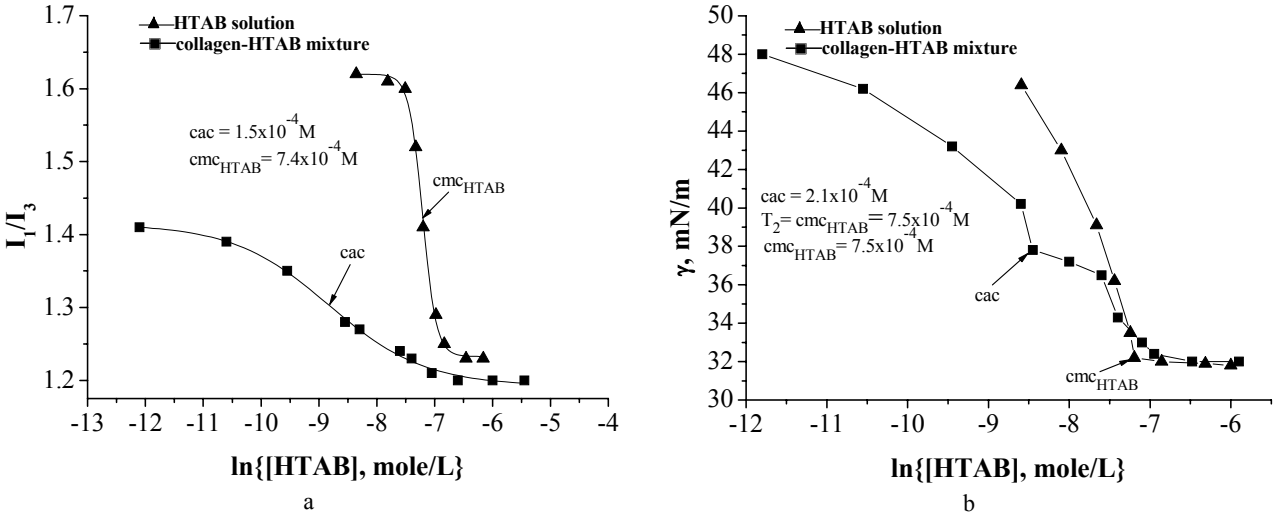


Fig. 13 – Dependence of (a) I_1/I_3 ratio and (b) surface tension versus the total HTAB concentration for the cationic surfactant solution and the collagen-HTAB system at pH 2.5 and 25^0C.

Such unexpected differences between the *cac*s for the collagen-HTAB and collagen hydrolyzate-HTAB systems at acidic pH could be determined by the differences in the conformational arrangements at molecular level for both collagen and collagen hydrolyzates. Whether the collagen possesses a relatively rigid conformation (triple helix), the collagen hydrolyzates have a great intramolecular flexibility at 25^0C (at which the investigations were performed), leading to an

overall random coil conformation more or less extended. Consequently, the access of HTAB cations to $–COO^-$ sites on the collagen triple helix could be more sterically hindered than in the case of the flexible polypeptide chains of H1 and H2. For the latters,
$–COO^-$ groups will be located at the distances at which the electrostatic contribution to the free energy of the system is lowest. Moreover, the larger net-positive charge of the compact collagen molecule also could shift the *cac* to HTAB concentration higher than for the collagen hydrolyzates. At the same time, the micropolarity in the immediate vicinity of the fluorescence probe measured at *cac* is clearly higher than that for the ordinary micelles of HTAB (Fig. 13a). This tendency was also observed in the case of the collagen-SDS system at pH 8.0 (Fig. 12a). Comparatively, the H1/H2-HTAB systems (at the same acidic pH) exhibit at *cac* a value of micropolarity almost equal to that measured for the normal HTAB micelles.

In the case of the saturation adsorption of HTAB onto collagen, the results are similar to those for the H1/H2-HTAB systems: the critical concentration T_2 is almost equal to the *cmc* of HTAB (Fig. 13b).

At basic pH (8.0), the collagen–HTAB system behaves rather similar to the collagen hydrolyzate-HTAB system at the same experimental conditions: (a) there is no satisfactory possibility to fit the fluorescence data (by the Boltzmann sigmoid function) in order to obtain a reliable value of *cac*, I_1/I_3 values revealing a low environment micropolarity compared with that for HTAB micelles at surfactant concentrations equal to or above its *cmc*; (b) the surface tension data indicate a possible *cac* value of $1.1x10^{-4}$ M, much higher than the *cac* obtained for the collagen hydrolyzate-HTAB system at pH 8.0 (probably due to the triple helical conformation of the collagen, as also explained above); (c) the critical concentration T_2 is almost equal to the *cmc* of HTAB (at the same experimental conditions) as it has been observed for all the polypeptide/collagen-HTAB systems.

The situation depicted here is graphically illustrated in figure 14.

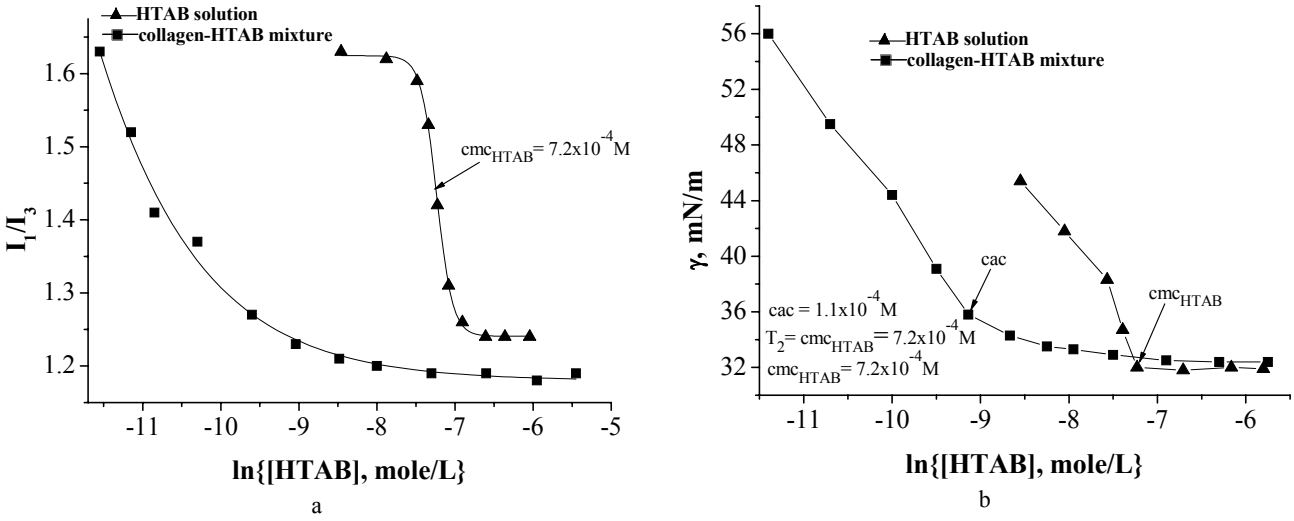


Fig. 14 – Dependence of (a) I_1/I_3 ratio and (b) surface tension versus the total HTAB concentration for the surfactant solution and the collagen-HTAB system at pH 8.0 and 25^0C.

The contradistinctions observed throughout the collagen hydrolyzate/collagen-HTAB, irrespective of pH, requires further investigations to bring into agreement the experimental data obtained at surfactant concentrations equal to or above its *cmc*: Py fluorescence exhibits a micropolarity obviously lower than that for the ordinary surfactant micelles whereas the surface tension data do not show any saturation adsorption of HTAB onto macromolecular component.

3. Possible mechanisms on the formation of the collagenous polypeptide-ionic surfactant complexes

In order to illustrate the probable picture on the formation of the collagenous polypeptide-ionic surfactant complexes it has been supposed that the primary structures of the two collagen hydrolyzates and the collagen itself are practically the same. Going from this standpoint, the amino acids

composition of the collagen type I extracted from skin of calf and rat (mean number of specified amino acid residues/1,000 amino acid residues: 30 Asp, 40 Glu, 50 Arg, 31 Lys, 2-3 His)[58] might lead to a IEP of 8-9, according to figure 6. As a matter of fact, this is confirmed by investigations on the pepsin solubilized calfskin collagen.[51] The isoelectric ranges of the collagen and the two hydrolyzates utilized are much below 8-9, which is certainly due to the chemical processes involved in the extraction and purification of collagen, on the one hand, and those in the obtaining of H1 and H2, on the other hand. Practically, the number of the amine groups (belonging especially to Arg and Lys residues) decreased with respect to carboxyl ones (from Asp and Glu residues). In this way, the isoelectric ranges were shifted to more acidic pHs. Under these considerations it is realistic to suppose that the number of the amine groups is approximately equal to that of the carboxyl ones for the investigated polypeptides and, depending on pH, the net molecular charge will be positive or negative (Fig. 6).

As it has been pointed out at items 1 and 2, even when the net charge of the macromolecular component has the same sign as for the ionic surfactant (polypeptide-SDS at pH 8.0; polypeptide-HTAB at pH 2.5), a significant number of charged groups with the opposite sign ($-NH_3^+$ at pH 8.0; $-COO^-$ at pH 2.5) make the favorable interaction between polypeptide and surfactant possible. If the net charge of the polypeptide has the opposite sign to that of the ionic surfactant, the electrostatic factor may induce a strong attractive interaction between the two components at lower surfactant concentrations and, in the case of SDS, lead to the formation of complexes with a low water-solubility (H1/H2-SDS at acidic pH) and even to a massive precipitation of protein (collagen-SDS at pH 2.5). The formation of low-soluble complexes using SDS takes place within a certain surfactant concentration range located between *cac* and *cmc*. Therefore, the polypeptide probably induces the ionic surfactant aggregation onto its chain before the polypeptide-ionic surfactant structure becomes insoluble. Obviously, there are no phase separations in any polypeptide-ionic surfactant systems in which both components carry an overall electric charge with the same sign, the complexes formed being water-soluble throughout the concentration range of surfactant.

Regarding the probable structure of the collagenous polypeptide-ionic surfactant aggregates, it depends on the molecular flexibility of polypeptide.

In the case of the two hydrolyzates, their high flexibility could be in accordance with structures of the complexes described by the necklace model.[27] At the same time, considering the description above-mentioned, the interaction between the collagen hydrolyzate and the ionic surfactant, depending on pH and surfactant concentration, could be depicted as shown in figure 15.

The stages III and IV in figure 15 are proposed only for the systems with SDS. The clarification of the aspects revealed by Py fluorescence and the surface tension measurements will bring further information on the possible structure of the H1/H2-HTAB complexes at surfactant concentration near and above its *cmc*. This clarification is required the more so, at basic pH, none of the collagen hydrolyzates lead to insoluble or low-soluble complexes within the concentration range of HTAB *cac*-*cmc*.

In the case of the collagen-surfactant mixtures, due to the triple helical structure of the protein (low intrachain flexibility at triple helix scale), the structure of the adsorption complexes cannot be as in the necklace model, but only as rod-like one on which the ionic surfactant clusters bind from place to place to the triple helix, at surfactant concentrations above *cac* and below *cmc*. At the saturation adsorption the micelle-like clusters of surfactant adsorbed onto the collagen molecule probably become more numerous and more bulky, resulting a final structure of rigid cylinder within which the triple helix is covered throughout its length by a bilayer of ionic surfactant. The sign of the net charge of this soluble collagen-surfactant complex is that of the surfactant charge. In figure 16 the formation of surfactant clusters bound to the collagen molecule as a function of the surfactant concentration is schematically illustrated.

Because of the same kind of reasons shown above for the hydrolyzate-HTAB systems, the stages III an IV in figure 16 are most suiting for the collagen-SDS pair, not for the collagen-HTAB.

Although no denaturing effect of the surfactant (anionic or cationic) on the collagen triple helix was observed at surfactant concentrations above its *cmc*, however the decrease of the denaturation temperature of the collagen type I in the presence of the ionic surfactant indicates the denaturing potential of the two ionic surfactants.[59]

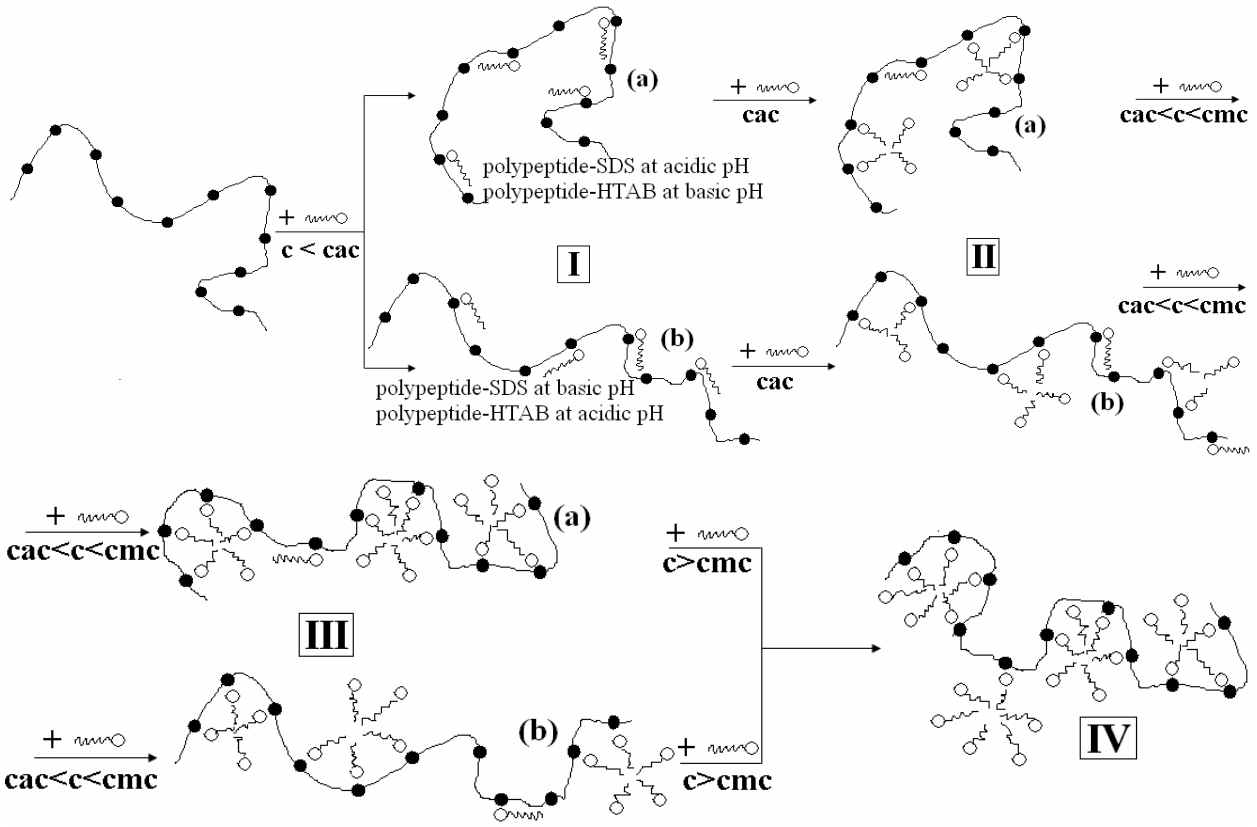


Fig. 15 – Scheme of a possible mechanism of flexible collagenous polypeptide-ionic surfactant interaction depending on pH and surfactant concentration.

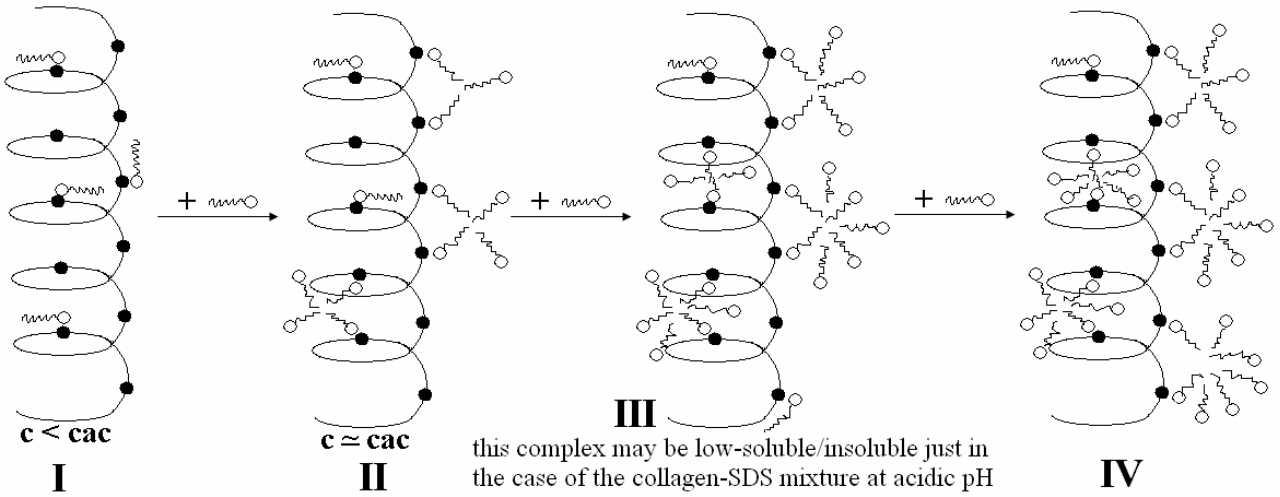


Fig. 16 – Depiction of collagen-ionic surfactant interaction as a function of surfactant concentration and pH (to simplify the scheme, a single helix was considered instead of triple helix for the native structure of collagen).

Both in figure 15 and 16, in order to simplify the scheme, only the potentially adsorption charged sites with the opposite sign to that of ionic surfactant were drawn. Consequently: (a) at pH 2.5, for the systems with SDS, only the positive charges ($-NH_3^+$) were marked. Thus, the presence of the other charges ($-COO^-$, hidden) will lead, within a certain concentration range of surfactant, to tight conformations of the flexible polypeptide chains (Fig. 15, I-IIIa) or even to a massive coagulation process of the collagen (Fig. 16, III); (b) at pH 8.0, for the systems with SDS, the picture contains merely the positive charges as well. Even though these positive charges are less numerous

than the negative ones (-COO^-), they still represent the sites onto polypeptide which favor the interaction with the anionic surfactant. The negative charges, uninserted in the picture, will expand the conformation of the flexible polypeptides (Fig. 15, I-IIIb) leading, along with the adsorbed surfactant, to high solubility complexes for all the systems hydrolyzate/collagen-SDS at pH 8.0; (c) at acidic pH (2.5), in the cases of the systems with HTAB only the negative charges of the polypeptide were graphically considered, the another positive ones (uninserted) causing a particular behavior described for the polypeptide-SDS systems at pH 8.0; (d) at basic pH (8.0), in order to highlight the interaction with HTAB, only the majority charged groups –COO^- of the polypeptide are drawn. By the contribution of the positive groups of the polypeptide, a behavior pretty similar to that of the systems with SDS at acidic pH is expected. Yet, the single great difference between these systems consists of the fact that the processes of polypeptide precipitation/coagulation do not occur at the HTAB concentrations up to *cmc*, but above it (the precipitate formation at the surfactant titration, Fig. 3).

EXPERIMENTAL

Materials. The collagen hydrolyzates (as white powder) and the calfskin collagen type I (as solution 0.85% w/v in acetic acid 0.1 M) were received from ICPI Bucharest. The freshly prepared stock solutions of the collagen hydrolyzates (by dissolving hydrolyzate powder in acetic acid 0.1 M) of 8% (w/v) together with the collagen solution were stored at cca 4^0C. The ionic surfactants, sodium dodecyl sulfate (99%+, Sigma Aldrich) and hexadecyl trimethylammonium bromide (>99%, Fluka) were used without further purifications. Pyrene (p.a. for fluorescence, Fluka), as fluorescence probe, was used as received to obtain a fresh stock solution (0.8×10^{-3} M) in anhydrous ethanol. The other reagents (analytical grade) were purchased from Reactivul-Bucharest.

Fluorescence measurements. The preparation of an aqueous solution of pyrene (cca 4.5×10^{-7} M) from its stock solution was achieved following a procedure presented elsewhere.[46] The pyrene concentration was fixed at 4×10^{-7} M throughout the systems investigated by the fluorescence probes method. The optimization of the pH values was done using glacial acetic acid (for acidic pHs) and aqueous sodium hydroxide solution 0.1 N (for basic pH). The absorption spectrum of pyrene (by UV-VIS spectrophotometer Jasco-V660) reveals three peaks within 250-350 nm, the most significant of them being localized at 334 nm. These patterns were observed for both the ethanol and n-hexane solution of pyrene. The values of I_1/I_3 (I_1 – intensity of the vibronic transition $S_1^{v=0} \rightarrow S_0^{v=0}$ at ≈373 nm; I_3 – intensity of the vibronic transition $S_1^{v=0} \rightarrow S_0^{v=1}$ at ≈384 nm)[40] were obtained from the emission fluorescence spectra of pyrene in the studied systems, recorded by a spectrofluorometer Jasco-6300 equipped with a thermostating module. The chosen excitation wavelength was 334 nm and the emission fluorescence spectra were recorded over the range 350-500 nm. The preliminary investigations performed with the pyrene-free polypeptide systems show a significant absorption only at the wavelengths below 250 nm. Moreover, none of the components (excepting pyren) of the systems exhibits fluorescence.

Surface tension measurements. The surface tension was measured by the droplet volume method. The employed equipment was assembled in our laboratory. This consists of a stainless steel cylinder (having a capillary channel of 1.341 mm diameter, oriented central along the cylinder), a 2 mL syringe with a constant diameter and a Teflon piston (Precision Analytical Syringe, SUPLECO, L.A., USA), a device with micrometer screw (for a precise determination of piston displacements), and a robust rack by which the entire ensemble is kept vertical and static. To eliminate the drop size variations during the evaporation, a glass phial of about 15 mL was attached tight on the downside of the steel cylinder. In this way, cca 7-8 mL of corresponding sample were put into the phial prior measurements to remain there during the experiment. At the same time, a side glass tube (which can be prolonged by a proper silicon flexible pipe) was welded to the glass phial in order to equilibrate the pressure between inside and outside of the phial. The measurements in the thermosetting regime were achieved by immersing the entire ensemble into a filled water polycarbonate vessel, the temperature of which was maintained at 25.0 ± 0.1^0C. The surface tension (γ) of a certain liquid system was obtained by the relationship:

$$\gamma = \frac{V \cdot (\Delta\rho) \cdot g}{r} \cdot F \qquad (4)$$

where V is the mean volume of the droplets formed (at the bottom end of the steel cylinder) and fallen (inside the phial) during the experiment, $\Delta\rho$ – the density difference between the studied system and air, g – the gravity acceleration, r – the external radius of the stainless steel capillary tube (2.744 mm), and F – a tabulated correction factor,[60] the value of which depends on $r/V^{1/3}$ ratio. On an average, for each set of measurement, a number of 8-10 droplets were considered, resulting an accuracy of ±0.1 mN/m for the surface tension determination.

Viscosity measurements. Capillary viscometry was used to obtain the intrinsic viscosities and then the molecular weights for the two collagen hydrolyzates and the collagen. In this respect, a suspended-level viscometer (Ubbelohde type, SCHOTT, Mainz) with a capillary constant K of 0.009056 mm^2/s^2 was employed. The measurements lead to kinematic viscosities (in mm^2/s or cstokes) directly by:

$$\nu = K(t - t^*) \qquad (5)$$

where ν is the kinematic viscosity, t – the efflux time (in seconds) of the liquid through the capillary of constant K, and t* - the kinetic energy correction (for a certain efflux time through a certain capillary), also referred to as the Hagenbach correction. The flow time measurements (with an accuracy ±0.01 s) were done by the Viscosity Measuring Unit Viscoclock, an electronic device which was interfaced to PC.

The kinematic viscosity is related to the specific viscosity (in equation (1)) as follows:

$$\eta_{sp} = \frac{\nu\rho}{\nu_0\rho_0} - 1 \qquad (6)$$

where ν and ρ represent the kinematic viscosity and the density, respectively, for the aqueous hydrolyzate/collagen systems studied (at the concentrations and the temperatures shown in figures 2 and 10), ν_0 and ρ_0 – the same quantities measured at the same temperatures for the solvents of the hydrolyzate/ collagen solutions (water at pH 4.0 and 37^0C for the hydrolyzate solutions; acetic acid 0.15 M at 24.8^0C for the collagen solutions). The densities of the hydrolyzate/collagen solutions were determined by a borosilicate glass picnometer (100 mL). The volume corrections for the picnometer at the working temperatures were made considering a coefficient of linear thermal expansion (α) of 4 μm $m^{-1}K^{-1}$ at 20^0C.[61] Supposing that the borosilicate glass is an exactly isotropic material, its coefficient of volumetric thermal expansion (β) is three times its coefficient of linear thermal expansion (β=3α) and the dependence between the corrected (V) and standard volume (V_0, 100 mL at 20^0C) of the picnometer was expressed by:

$$V = V_0(1+\beta\cdot\Delta T) \quad (7)$$

where ΔT is the difference between the standard and working temperature.

UV-Circular dichroism measurements. In order to obtain the circular dichroism spectrum for the collagen, a solution of 0.15% (w/v) in acetic acid 0.1 M was prepared. Using a spectropolarimeter Jasco J-810 with a square quartz cuvette (Suprasil, path length 0.02 cm) for UV domain, the following working parameters are set: wavelength range 250-190 nm; scanning speed of spectra - 50 nm/min with 0.2 nm pitch; number of spectra acquisition - 4; working temperature - 23^0C (room temperature); proper flow injection of nitrogen (purity 5.5) to eliminate oxygen absorption within wavelength range 210-190 nm.

Electron microscopy measurements. To visualize the collagen at molecular scale, the samples were prepared according to the mica sandwich-rotary shadowing technique.[62-66] Shortly, this technique consists in the obtaining of the metallic replica of the collagen molecules prior adsorbed onto an atomically flat surface. Mica foils freshly cleaved were used as flat surfaces. In this way, a drop of collagen solution (≈5 μL) is placed centrally on the one newly cleaved mica surface. The other freshly surface of mica is then gently laid on top resulting a sandwich packing with a solution middle layer of about 10 μm thick.[62] The system is kept rest 5-10 minutes to allow the adsorption of the collagen molecules on the mica substrate then the two pieces are separated, placed on a stage with the wet faces upward and dried in vacuum. After that, the metallic replica of the mica surfaces with the adsorbed collagen molecules were realized by vacuum evaporation of a heavy metal (Pt, Au, Pd, Cu). To visualize, by the replica formation, the collagen adsorbed on the mica substrate, a proper geometrical arrangement inside the chamber of vacuum evaporator is required. Thus, the distance between the sample to be coated and the source of the metal vapors has to be of about 10 cm and the angle between the line that unite the metal vapor source and the sample and the surface of sample has to be lower than 10^o. Then, to avoid the shadowing effect and the hiding of some collagen molecules, the samples stage with the described geometry must be rotated with a constant speed (1-2 cycles per second) during the metal evaporation (by resistive heating of tungsten filament). The metal replicas resulted in this way are reinforced by graphite deposition (by electric arc) at high vacuum and an incidence angle of 90^o. The detachment of the metallic coatings from the mica surfaces is performed by carefully immersion of each piece of mica in distilled water at an immersion angle of about 20^o so that the metallic film is pointed upward. The detachment is much improved by prior keeping the mica samples above a vessel filled with acetic acid 1M (all enclosed into a desiccator).[62] The pieces of replicas floating off onto distilled water are transferred on the cooper grids and then left to air dry. After these procedures the metallic replicas are ready to expose to the electron beam in the microscope.

One of the most important aspects of this experimental algorithm is the choice of a suitable concentration of collagen solution that the estimated number of molecules adsorbed on the inner surfaces of the mica sandwich is 20-30/μm^2 (an optimum number to the best visualization of replica patterns). Going from this prerequisite and considering the mean surface area of the mica foils of 4-5 cm^2, the inner layer of solution into the sandwich packing of about 10 μm thick[62] and the molecular size of the collagen (≈300 nm length, ≈$3x10^5$ a.m.u. – molecular weight), a collagen solution of $1.5x10^{-4}$ % (w/v) has been considered as very good initial sample to obtain a roughly surface density of 15 collagen molecules/μm^2. At the same time, a uniform distribution of the collagen molecules adsorbed on the mica substrate can be attained by the use of anhydrous glycerin to a final proportion of 70% in the collagen solution.[62,63] The samples prepared as described above were dried for 3 hours and subsequently coated by Cu (purity 99.99%, 5^o – deposition angle) and graphite (90^o – incident angle) evaporation employing a high vacuum evaporator Jeol-JEE 4C ($5x10^{-6}$ torrs).

The samples visualization was performed by transmission electron microscopy using a JEOL TEMSCAN 200CX microscope at the working acceleration voltage of 200 kV.

CONCLUSIONS

The investigation of the collagenous polypeptide-ionic surfactant systems performed at pHs below and above the isoelectric range of the polypeptide revealed significant attractive interactions between the two components at surfactant concentrations much below *cmc* throughout the cases studied. In this respect, Py fluorescence and the surface tension data are conclusive and in a pretty good agreement.

The onset of the ionic surfactant association induced by the polypeptide (*cac*) is best observed for the systems consisted of the charged polypeptide and the ionic surfactant having the charge with the same sign to that of the net charge of macromolecular component. For all these systems the steady-state fluorescence data are satisfactory consistent with the surface tension results. The electrostatic factor responsible for the polypeptide-surfactant complexation in the mentioned systems is represented by the significant proportion of the ionic groups carrying the charge with the opposite sign to that of the ionic surfactant (cca 74% of carboxyl groups as carboxylate form at pH 2.5; about 91% of amine groups as ammonium form at pH 8.0).

In the polypeptide-surfactant systems in which the surfactant possesses an opposite charge to the net charge of the polypeptide, there are several important differences between the mixtures with SDS and those with HTAB. Thus, in the case of the polypeptide-SDS systems at acidic pH, there is a good consistence between the results of the two experimental methods (fluorescence and surface tension) merely for the collagen hydrolyzate with the lower molecular weight (H1). For the hydrolyzate H2, only the surface tension data seems to exhibit the onset of SDS aggregation, while for the collagen the data come from both fluorescence and surface tension do not indicate any value for *cac*. At the same time, all the polypeptide-SDS systems at acidic pH show a concentration domain of surfactant located between *cac* and *cmc* within which the polypeptide-anionic surfactant complexes with low solubility are supposed to be formed. In the case of the collagen, a massive precipitation of protein takes place within such a domain of SDS concentration. Regarding the polypeptide-HTAB at basic pH, the experimental data (fluorescence and surface tension) offered approached *cac* values only for the hydrolyzate H1. For the hydrolyzate H2 and collagen, *cac*s were obtained merely by the surface tension measurements. Moreover, throughout the HTAB concentrations ranged between *cac* and *cmc*, the formation of the insoluble or low-soluble polypeptide-surfactant complexes was not observed.

The saturation adsorption in the systems studied exhibits particular characteristics for both SDS and HTAB. For all the systems with SDS, the critical concentration T_2 (from the surface tension data) is higher than *cmc* irrespective of pH. In the case of all the systems with HTAB, however, the surface tension data reveal a T_2 value almost equal to *cmc* of the cationic surfactant. This would be equivalent to the lack of HTAB adsorption onto the polypeptide component which is in contradistinction with Py fluorescence data. The achievement of further investigations in this respect is a normal requirement.

In regard to the flexible polypeptides (H1 and H2), it is considered that the suited model able to describe the interaction with the ionic surfactants is the necklace model, whereas the rigid rod model is considered as the proper model for the collagen in its interaction with the ionic surfactants.

The use of these polypeptide components in several cosmetic formulations like soaps and shampoos leads to a marked decrease of the effective critical micelle concentration of ionic surfactants much below their *cmc*s. From this point of view, the proportion of the ionic surface-active component in such cosmetics could be substantially lowered together with enhancing mildness action of these products toward skin.

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