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Free amino group content of serum albumin microcapsules. III. A study at low pH values

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Abstract

Microcapsules were prepared by interfacial cross-linking of human serum albumin (HSA) with terephthaloyl chloride (TC). Determination of free amino groups was performed on lyophilized microcapsules with trinitrobenzenesulfonic acid (TNBS). In two series of assays conducted with phosphate buffers, pH 7.4 and 8, respectively, microcapsules exhibited a high $-NH_2$ content (> 330 μmol/g dry weight), with only a slight decrease when raising TC concentration from 2.5 to 5% TC (w/v) and/or reaction time from 30 to 60 min. When the phosphate buffer, pH 7.4 (ionic strength (μ): 0.40), was replaced by an acetate buffer, pH 7.4 (μ: 0.66), supplemented with 0.125 or 0.25 M $CaCl_2$, microcapsule $-NH_2$ content was reduced from 346 to 134 and 168 μmol/g, respectively. Control batches prepared with the acetate buffer without adding $CaCl_2$ also had a reduced $-NH_2$ content (182 μmol/g), while a higher value (302 μmol/g) was found for microcapsules prepared using a phosphate buffer with a 0.66 ionic strength. Finally, a sharp decrease in $-NH_2$ content was also found for microcapsules prepared from acetate buffers at pH 5.9 (174 μmol/g) and 6.8 (158 μmol/g), as compared with microcapsules prepared from phosphate buffers at the same pH values (438 and 388 μmol/g, respectively).

Keywords: Microcapsule; Human serum albumin; Interfacial cross-linking; Terephthaloyl chloride; Amino group determination; Trinitrobenzenesulfonic acid

1. Introduction

This work is part of a series of structural studies that we have been performing on microcapsules prepared by interfacial cross-linking of human serum albumin (HSA) with terephthaloyl chloride (TC). The aim was to determine which functional groups of the protein were involved in the formation of the membrane and to what extent, depending on the reaction parameters. We have been investigating the effect of independent variations of reaction parameters on structural changes in HSA by means of two complementary methods, namely Fourier transform infrared (FT-IR) spectroscopy (Lévy et al., 1991; Lévy et al., 1994; Lévy et al., 1995), and determination of the free amino group content of microcapsules with trinitrobenzenesulfonic acid (TNBS) (Edwards-

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Lévy et al., 1993; Edwards-Lévy et al., 1994). Along these studies, it was shown that increasing the reaction pH from 5.9 to 11 resulted in a progressive acylation of hydroxy and carboxylate groups to esters and anhydrides, respectively (Lévy et al., 1991), while the microcapsule $-NH_2$ content progressively decreased from more than 450 μmol/g dry weight to 50 μmol/g (Edwards-Lévy et al., 1993). These structural changes of HSA upon acylation were most pronounced from pH 9 and were shown to correspond to a decrease in microcapsule mean size and to modifications of the membrane surface, made rough. In two other studies conducted at a constant pH of 9.8, TC concentration (with 30 min reaction time), and reaction time (with 2.5% TC) were varied independently. Highly cross-linked membranes were obtained even with short reaction times or low TC concentrations, as reflected by the NH_2 content which did not exceed 154 μmol/g (Edwards-Lévy et al., 1994), and by the high amount of esters and anhydrides which were observed in the FT-IR spectroscopic studies (Lévy et al., 1994; Lévy et al., 1995).

The present study is devoted to microcapsules prepared at low pH values (≤ 8). The purpose of the first part of the work was to determine whether highly cross-linked membranes with low $-NH_2$ contents would be obtained at pH 8 or 7.4 by increasing the reaction time from 30 to 60 min and/or the TC concentration from 2.5 to 5% (w/v). In the following part of the work, which was conducted at pH 7.4, variations were introduced in the composition of the aqueous phase. The HSA solution was supplemented with $CaCl_2$, the phosphate buffer being replaced by an acetate buffer in order to avoid precipitation with the calcium salt. The aim of these experiments was to induce an unfolding of the protein, so that buried acylable groups would be exposed at the interface. As a matter of fact, HSA has been shown to undergo conformational changes around neutral pH in the presence of calcium ions (Wilting et al., 1979). These changes are known to be comparable to those observed when increasing the pH from 7 to 9, which are commonly referred to as the neutral-to-base (N-B) transition (Leonard et al., 1963). Otherwise, increasing ionic strength has been reported to favour the N-B transition of serum albumin (Wilting et al., 1979), and to induce unfolding of proteins at interfaces (Cumper and Alexander, 1950). In order to investigate the role of the ionic strength, which was lower in the phosphate buffer (μ: 0.40) than in the acetate buffer (μ: 0.66), control experiments were then performed in this study with microcapsules prepared with the acetate buffer without adding calcium, and also with microcapsules prepared with a second phosphate buffer having the same ionic strength (0.66) as the acetate buffer. Finally, the study was extended to microcapsules prepared at pH 5.9 and 6.8, in two parallel series of assays using either acetate buffers or phosphate buffers.

2. Materials and methods

Materials and methods have been described in details elsewhere (Lévy et al., 1991; Edwards-Lévy et al., 1993).

2.1. Preparation of the microcapsules

Briefly, microcapsule batches were prepared from a 20% (w/v) HSA buffered solution. The HSA solution was emulsified in an organic phase and a TC solution in the organic phase was added to the emulsion. After reaction, the resulting microcapsules were centrifuged, washed and lyophilized.

In the first series of experiments, HSA was dissolved in a phosphate buffer, pH 7.4 or 8 (μ: 0.40 or 0.30, respectively). Variations of parameters were performed as follows: 2.5% (w/v) TC was combined with a 60-min reaction time, and 5% TC with 30- or 60-min reaction times. The results were compared with those obtained in a previous study with 2.5% TC and 30-min reaction time using the same batch of HSA (Edwards-Lévy et al., 1993).

In the following experiments, all microcapsules were prepared with a new batch of HSA obtained from the same supplier (CTS, Strasbourg, France). For studying the influence of calcium ions, the HSA was dissolved in an acetate buffer, pH 7.4 (μ: 0.66), supplemented with $CaCl_2$ at a

final concentration of 0.125 or 0.25 M (final ionic strength: 1.04 and 1.41, respectively). Reaction time (30 min) and TC concentration (2.5% TC) were kept constant. Control batches were prepared with the acetate buffer without adding calcium, and with the phosphate buffer, pH 7.4 (because of the change in the batch of HSA). Moreover, microcapsules were prepared with a second phosphate buffer, pH 7.4, having the same ionic strength as the acetate buffer (0.66). In the third part of the study, devoted to the influence of the buffer (phosphate or acetate) at pH < 7.4, microcapsules were prepared with 2.5% TC and 30-min reaction time, using either phosphate buffers, pH 5.9 or 6.8, or acetate buffers, pH 5.9 or 6.8 (μ: 0.40; 0.40; 0.72; 0.66, respectively).

2.2. Determination of microcapsule free amino groups

The TNBS method (Edwards-Lévy et al., 1993) was applied to samples of 10 mg of lyophilized microcapsules. Mean values were calculated from four determinations: two samples were analyzed per batch and two batches of microcapsules were examined for each value of the varied parameters.

3. Results and discussion

3.1. Study at pH 7.4 and 8: influence of reaction time and TC concentration on microcapsule free amino groups

Table 1 and Table 2 display the values of microcapsule $-NH_2$ content as a function of reaction time and TC concentration, for the two series obtained with phosphate buffers, pH 7.4 (Table 1) and pH 8 (Table 2), respectively.

Under all conditions, microcapsules exhibited high contents in free amino groups, exceeding 330 μmol/g. As expected, the highest value was that obtained at pH 7.4 with 2.5% TC and 30-min reaction time. At this pH of 7.4, a decrease in $-NH_2$ content was observed when increasing the time or the TC concentration. No significant additional decrease was observed with 5% TC when prolonging the reaction time to 60 min. At pH 8, two groups of results could be distinguished corresponding to 2.5 and 5% TC, respectively, the reaction time having no apparent effect on the $-NH_2$ content.

Table 1
Microcapsule NH_2 content as a function of reaction time and TC concentration (reaction pH: 7.4)

	TC concentration (%)							
	2.5				5			
	30 min		60 min		30 min		60 min	
Batch	1	2	3	4	5	6	7	8
NH_2 content	437	424	352	328	384	344	336	400
(μmol/g)	429	436	344	328	368	360	368	328
Mean value	431.5[a]		338		364		358	

[a]From a previous study (Edwards-Lévy et al., 1993).

These results again illustrate the important role of reaction pH in interfacial cross-linking of proteins. Loosely cross-linked membranes were obtained when using phosphate buffers at pH 7.4 or 8, even with a high TC concentration and/or a prolonged reaction time. Variations of the reaction parameters only resulted in slight variations in microcapsule $-NH_2$ content. However, TC concentration exerts an influence, as raising the concentration to 5% resulted in an even more efficient acylation of HSA amino groups. Concerning the reaction time, no effect was observed at pH 8 when prolonging the time from 30 to 60 min, which is in agreement with the results of our previous study conducted at pH 9.8, giving 30

Table 2
Microcapsule $-NH_2$ content as a function of reaction time and TC concentration (reaction pH: 8)

	TC concentration (%)							
	2.5				5			
	30 min		60 min		30 min		60 min	
Batch	1	2	3	4	5	6	7	8
$-NH_2$ content	432	395	376	432	352	360	376	344
(μmol/g)	432	389	464	432	384	400	352	376
Mean value	412[a]		426		374		362	

[a]From a previous study (Edwards-Lévy et al., 1993)

min as the optimal value for the interfacial acylation of HSA amino groups (Edwards-Lévy et al., 1994).

In the case of microcapsules prepared at pH 7.4, the observed decrease in $-NH_2$ content when raising the reaction time to 60 min with 2.5% TC might be accounted for by a late conformational change of HSA, exposing buried acylable groups to the outside. As a matter of fact, microcapsules prepared at pH 7.4 would represent a special case, as we had previously suggested (Edwards-Lévy et al., 1993), the denaturation of HSA being minimal at physiological pH. A long exposure to the solvent at the interface together with acylation itself would then progressively change this physiological state into the same denatured state as that obtained within a shorter time when using higher TC concentrations or higher pH values.

3.2. Study at pH 7.4: effect of calcium ions and influence of the buffer on microcapsule free amino groups

Table 3 displays the values of microcapsule $-NH_2$ content as a function of the composition of the buffered aqueous phase at pH 7.4.

As expected, addition of calcium to the aqueous phase (acetate buffer, batches 7–10) resulted in a sharp decrease in microcapsule $-NH_2$ content, as compared with the value (346 μmol/g) obtained with the phosphate buffer (μ: 0.40, new control batches 1 and 2). The lowest value was obtained with 0.125 M Ca^{2+} (134 μmol/g). Raising the calcium concentration to 0.25 M, rather than inducing an additional decrease, resulted in an increase in free amino groups (168 μmol/g).

Concerning control batches prepared without adding calcium, a marked decrease in free amino groups was also observed for microcapsules prepared with the acetate buffer (182 μmol/g, batches 5, 6), while the decrease was less pronounced in batches prepared with a phosphate buffer having the same ionic strength (0.66) as the acetate buffer (302 μmol/g, batches 3, 4).

Considering the effect of calcium ions, the results suggest that an optimal concentration would be required for a maximal acylation of amino groups. The higher $-NH_2$ content obtained with 0.25 M Ca^{2+} might be accounted for by a decrease in HSA solubility impairing the formation of the interfacial film, as suggested by the unusual granulous aspect of the microcapsule content as observed by light microscopy. However these experiments do not allow to conclude a specific effect of calcium ions. As a matter of fact, the results obtained in the control assays performed without calcium demonstrate that a decrease in microcapsule free amino groups can be obtained at pH 7.4 by simply raising the ionic strength of the buffer, the phenomenon being attributed to conformational changes in HSA, exposing acylable groups. This mechanism is then assumed to participate in the decrease in $-NH_2$ content observed with the batches prepared in the presence of calcium, for which the aqueous phase had the highest ionic strength.

Otherwise, for a given ionic strength, the composition of the buffer was shown to have an important influence, as reflected by the sharp decrease in $-NH_2$ content (182 μmol/g) observed

Table 3
Microcapsule $-NH_2$ content as a function of the composition of the aqueous phase at pH 7.4 (30 min; 2.5% TC)

Buffer	Phosphate	Phosphate	Phosphate	Phosphate	Acetate	Acetate	Acetate	Acetate	Acetate	Acetate
Calcium	-	-	-	-	-	-	0.125 M	0.125 M	0.25 M	0.25 M
Ionic strength	0.40	0.40	0.66	0.66	0.66	0.66	1.04	1.04	1.41	1.41
Batch	1	2	3	4	5	6	7	8	9	10
$-NH_2$ content	352	336	296	296	176	192	128	160	176	176
(μmol/g)	352	344	296	320	176	184	112	136	168	152
Mean value	346		302		182		134		168	

Table 4
Influence of the buffer on microcapsule $-NH_2$ content at pH 5.9 and 6.8 (30 min; 2.5% TC)

Reaction pH	5.9	5.9	5.9	5.9	6.8	6.8	6.8	6.8
Buffer	phosphate	phosphate	acetate	acetate	phosphate	phosphate	acetate	acetate
Ionic strength	0.40	0.40	0.72	0.72	0.40	0.40	0.66	0.66
Batch	1	2	3	4	5	6	7	8
$-NH_2$ content	440	440	160	168	376	376	144	144
(μmol/g)	440	432	184	184	384	416	152	192
Mean value	438		174		388		158	

with the acetate buffer as compared with a phosphate buffer having the same ionic strength (302 μmol/g). This difference could not be attributed to a more efficient neutralization of the released HCl resulting in higher amounts of unprotonated $-NH_2$, since the buffer capacity of the phosphate buffer was about 4 times higher than that of the acetate buffer.

This unexpected result prompted us to carry out comparative experiments with microcapsules prepared at lower pH values (5.9 and 6.8) with acetate and phosphate buffers, respectively.

3.3. Study at pH 5.9 and 6.8: influence of the buffer on microcapsule free amino groups

Table 4 displays the values of microcapsule $-NH_2$ content as a function of the composition of the buffer at pH 5.9 and 6.8.

Replacing the phosphate buffers by acetate buffers provided microcapsules with lower $-NH_2$ contents (174 and 158 instead of 438 and 388 μmol/g for pH 5.9 and 6.8, respectively), as it had been observed at pH 7.4. As discussed before, the higher ionic strength of the acetate buffers (Table 4) presumably participates in the phenomenon. However, the sharp decrease in free amino groups suggests the involvement of another mechanism, which would facilitate $-NH_2$ acylation.

It should be pointed out that these differences in microcapsule $-NH_2$ content were accompanied by morphological changes as observed by scanning electron microscopy (Andry and Lévy, unpublished data). 'Acetate microcapsules' exhibited a rough surface, while 'phosphate microcapsules' had a smooth membrane. According to previous observations (Lévy et al., 1995), the changes in microcapsule morphology reflect important structural changes in HSA, the roughness of the membrane being a signal for highly cross-linked membranes. A comparative study using FT-IR spectroscopy would certainly help to explain the origin of the differences between 'acetate' and 'phosphate' microcapsules, by giving complementary information on the involvement of hydroxy and carboxy groups of HSA in the membrane.

4. Conclusions

This study brings information on several factors influencing the degree of cross-linking of protein microcapsules when using buffers with low pH values.

In the first part conducted with phosphate buffers, only slight variations were observed in microcapsule free amino groups at pH 7.4 or 8, when raising the reaction time or the TC concentration. Loosely cross-linked membranes with high $-NH_2$ contents were obtained under all conditions.

On the other hand, variations in the composition of the aqueous phase were shown to greatly influence interfacial acylation of HSA amino groups. Microcapsule $-NH_2$ contents were decreased by increasing the ionic strength of the buffer, even without adding calcium. Moreover, comparative experiments conducted with phosphate and acetate buffers having the same ionic strength revealed an interesting effect of acetate buffers, allowing an intensified acylation of amino groups at pH 7.4, 6.8 and even 5.9.

This finding appears of great interest as it allows to increase the degree of cross-linking of the protein without raising the reaction pH as is usually done. This makes it possible to take advantage of interesting changes in microcapsule properties related to the intensified acylation, such as an improvement in the mechanical resistance of the membrane or a slower enzymatic degradation, while the reaction conditions become compatible with the encapsulation of materials needing a neutral or slightly acidic environment.

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