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Determination of free amino group content of serum albumin microcapsules: II. Effect of variations in reaction time and in terephthaloyl chloride concentration

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Summary

Microcapsules were prepared through interfacial cross-linking of human serum albumin (HSA) using terephthaloyl chloride (TC) at a constant pH of 9.8. Variations were performed in reaction time (for a 2.5% w/v TC concentration) and in TC concentration (for a 30 min reaction time). Determination of free amino groups was performed on lyophilized microcapsules by means of a back titration method using trinitrobenzenesulfonic acid (TNBS). When prolonging the reaction time from 2 to 30 min, microcapsule -NH_2 content progressively decreased from 154 to 60 μmol/g dry weight. These results were in good agreement with those of a previous study conducted using Fourier transform infrared spectroscopy. A slight recovery of free -NH_2 was observed after 60 and 120 min. Increasing TC concentration from 1 to 5% (w/v) resulted in a progressive decrease in microcapsule -NH_2 content from 99 to 38 μmol/g.

Introduction

This work takes place in the realm of structural studies that we have been performing on microcapsules prepared through interfacial cross-linking of human serum albumin (HSA) using terephthaloyl chloride (TC). In order to investigate the effect of independent variations of reaction parameters on structural changes in HSA, we have been making use of two complementary methods, namely Fourier transform infrared (FT-IR) spectroscopy (Lévy et al., 1991, 1993), and determination of the free amino group content of microcapsules by means of trinitrobenzenesulfonic acid (TNBS) (Edwards-Lévy et al., 1993).

In a recent work, we showed that increasing the reaction pH from 5.9 to 11, for constant reaction time (30 min) and TC concentration (2.5% w/v), resulted in a progressive decrease in microcapsule free amino groups from more than 400 μmol/g dry weight to under 110 μmol/g from pH 9, reflecting the progressive acylation of

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amino groups in HSA (Edwards-Lévy et al., 1993). These changes were shown to parallel the progressive acylation of other functional groups of HSA that was observed using FT-IR spectroscopy upon raising the reaction pH (Lévy et al., 1991). Increasing amounts of esters were shown to be formed from hydroxylamino acids, while a more unexpected formation of anhydrides from carboxylate groups was demonstrated in the study. A correlation was also found between structural changes of the protein and variations in microcapsule morphology and size. Small-sized microcapsules (diameters ≤ 15 μm) with a rough surface ('type I' microcapsules) were shown to correspond to low -NH_2 contents and high amounts of esters and anhydrides in the membrane, whereas large-sized ('type II') microcapsules (diameters: 30–40 μm) with a smooth membrane were found to correspond to high -NH_2 contents and low amounts of esters and anhydrides.

The purpose of the present study was to explore the influence of two more parameters on microcapsule -NH_2 content with the reaction pH fixed at pH 9.8, namely, the reaction time (for a constant 2.5% w/v concentration) and the TC concentration (for a fixed 30 min reaction time). The changes in microcapsule free amino groups were compared with those of the previous pH series of experiments. Moreover, the influence of reaction time on the acylation of -NH_2 groups was compared with the effects of this parameter on spectral changes that we recently observed in a parallel FT-IR study (Lévy et al., 1993). Finally a correlation was found with microcapsule morphology and size, as in our previous pH series of assays.

Materials and Methods

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

Preparation of the microcapsules

Variations of reaction parameters were performed as follows. In the first series of experiments devoted to the reaction time, microcapsule batches were prepared from a 20% (w/v) HSA solution in a buffer pH 9.8. The HSA solution was emulsified in an organic phase and a 2.5% (w/v) TC solution in the organic phase was further added to the emulsion, which started the polycondensation step. The reaction time was varied from 2 min to 5, 10, 15, 30, 60 and 120 min. The resulting microcapsules were centrifuged, washed and lyophilized. Another series of batches was prepared using a constant 30 min reaction time and TC concentrations increasing from 1.0 to 1.25, 2.5 and 5% (w/v).

Determination of microcapsule free amino groups

The TNBS method (Edwards-Lévy et al., 1993) was applied to lyophilized microcapsules. Briefly, 10 mg of microcapsule powder was incubated with an excess of TNBS. After 1 h reaction, the medium was filtrated. In a second step, the excess of TNBS was determined in the filtrate using an

TABLE 1

Microcapsulte -NH_2 content as a function of reaction time (pH, 9.8; TC concentration, 2.5%)

	Reaction time													
	2 min		5 min		10 min		15 min		30 min		60 min		120 min	
Batch	1	2	3	4	5	6	7	8	9	10	11	12	13	14
-NH_2 content	148	144	96	112	88	96	68	80	59	48	72	80	96	100
(μmol/g)	164	160	104	80	104	72	64	80	56	77	75	72	100	100
Mean value	154		98		90		73		60		75		99	

incubation with an excess of valine. The resulting TNP-valine derivative was determined spectrophotometrically. 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.

Results and Discussion

Influence of reaction time on microcapsule free amino groups

Table 1 displays the values of microcapsule -NH_2 content as a function of reaction time. As early as 2 min after addition of TC, the amount of free amino groups was found to be low (154 μmol/g dry weight), as compared with the high values ($\approx$ 400 μmol/g) that had been observed in the pH series of experiments for microcapsules prepared at pH $\leq$ 8 using a 30 min reaction time. This result provides evidence of a rapid acylation of HSA amino groups at a high reaction pH. This observation is in agreement with the high rates of acylation that have been reported in the literature in the case of interfacial polycondensation of diamines with acid dichlorides at high pH values (Bradbury and Crawford, 1977). A further decrease in free amino groups was still noted when prolonging the reaction time from 2 to 30 min, with a marked fall observed after 5 min, and a minimal value of 60 μmol/g reached after 30 min.

Surprisingly, no additional decrease in microcapsule -NH_2 content occurred upon prolonging the reaction time to 60 and 120 min. In contrast, the amount of free amino groups was shown to slightly increase.

Fig. 1 compares the results of the initial 60 min period with the spectral changes observed using FT-IR spectroscopy and with the corresponding modifications in microcapsule size (Lévy et al., 1993). Concerning spectral changes, the initial period of 2 min must be considered separately. As a matter of fact, the microcapsule content in esters and carboxylates was shown to decrease during this early stage, as a result of an immediate interfacial dissolution of contaminating lipids of HSA. However, a significant increase in the β-sheet band area (at 1624 cm^{-1}) was noted as compared with pure HSA. This observation was assumed to reflect important interchain H bonding following the acylation of HSA amino groups during the 2 min period. The present study then confirms the involvement of HSA amino groups in the membrane after 2 min. Examination of the corresponding microcapsule size shows a large diameter (31 μm), corresponding to type II loosely cross-linked microcapsules, which otherwise were found to characteristically exhibit a smooth surface, as shown by scanning electron microscopy.

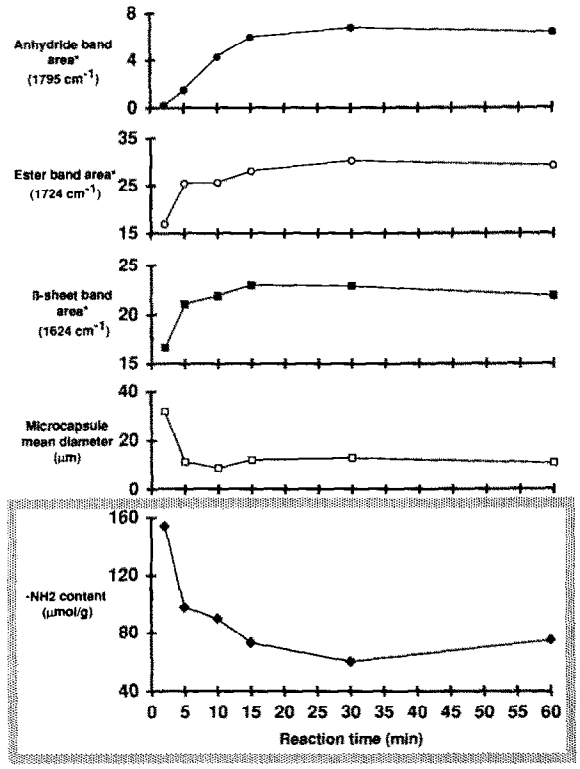

Fig. 1. Influence of reaction time on the involvement of functional groups of HSA in microcapsule membrane and concurrent changes in microcapsule mean size. * From FT-IR spectroscopic studies (Lévy et al., 1993): arbitrary units.

Prolonging the reaction time to 5 min resulted in important structural changes. Paralleling the intensified acylation of amino groups, the in-

creased formation of esters and anhydrides was observed. These changes, which were accompanied by a decrease in the carboxylate band area, reflected the acylation of the other functional groups of HSA, i.e., hydroxy and carboxylate groups (in the form of esters and anhydrides, respectively), which started at this time. The intensified acylation of HSA resulted in a sharp increase in the β-sheet content.

Examination of microcapsule size showed a significant decrease to under 15 μm after 5 min, corresponding to the typical aspect of highly cross-linked membranes with a rough surface. These type I microcapsules were also observed for all reaction times while their surfaces were shown to be progressively rougher.

All functional groups of HSA continued to be acylated during the following period of time as shown by a progressive increase in ester, anhydride and β-sheet band areas, accompanied by a decrease in the free $-NH_2$ content. However, whereas the formation of anhydrides appeared to regularly increase during the initial 15 min, the microcapsule ester, β-sheet and $-NH_2$ contents showed more irregular variations, smaller changes occurring during the 5–10 min period, as compared with the 2–5 and 10–15 min periods.

Maximal acylation of all functional groups appeared to be reached after 30 min. As a matter of fact, when prolonging the reaction time to 60 min, a slight decrease in the band areas corresponding to esters, anhydrides and β-sheet was observed, paralleling the slight increase in free amino groups. It should be pointed out that these minor variations, which were observed after 60 min, were not accompanied by an increase in microcapsule size (mean size: 10.75 ± 0.27 μm), unlike what had been observed, for example, after soaking microcapsules in a slightly alkaline buffer, which disrupted anhydride bonds together with part of the ester bonds of the membrane (Lévy et al., 1991). Nor could we observe a significant increase in size for microcapsules prepared using a 120 min reaction time in this study. Their mean size was close to 15 μm (14.97 ± 0.37 μm), while scanning electron microscopy still revealed a rugged membrane, similar to that after 60 min reaction.

However, the slight deacylation of the various functional groups of HSA, appearing for prolonged reaction times, was unexpected. It might be accounted for by a late hydrolysis reaction of the newly formed bonds in the membrane, that would be initiated by local environment changes due to an intense unfolding of HSA. As a matter of fact, proteins are known to undergo important conformational changes at interfaces (Cumper and Alexander, 1950), the extent of which may increase under the influence of a high pH and of acylation itself (Chang and Sun, 1978).

Otherwise, it cannot be excluded that a late unfolding of HSA might have an additional effect in exposing new amounts of unreacted amino groups thereby made accessible to TNBS.

These results then demonstrate that at pH 9.8, most important structural changes in HSA occurred within the 5–30 min period, during which the three kinds of functional groups of the protein were progressively acylated.

Influence of TC concentration on microcapsule free amino groups

Table 2 displays the values of microcapsule $-NH_2$ content as a function of TC concentration. It should be noted that stable microcapsules could be prepared using only 0.5% TC. However, their $-NH_2$ content could not be evaluated, due to the formation of numerous aggregates which would have impaired the determination.

The results show that even with 1% TC, the amount of free amino groups was low after 30 min reaction (≈ 100 μmol/g). As expected, increasing TC concentration resulted in a further

TABLE 2

Microcapsule $-NH_2$ content as a function of TC concentration (pH, 9.8; reaction time, 30 min)

	TC concentration % (w/v)							
	1		1.25		2.5		5	
Batch	1	2	3	4	5	6	7	8
$-NH_2$ content (μmol/g)	100	104	64	88	59	48	32	32
	96	96	80	88	56	77	56	32
Mean value	99		80		60		38	

decrease in -NH_2 content with a minimal value of 38 μmol/g for 5% TC. These results provide evidence of a high degree of acylation of HSA amino groups at pH 9.8, even for relatively low TC concentrations. They also demonstrate the influence of the TC concentration on the reaction, which is in agreement with literature data concerning the interfacial acylation of diamines with acid dichlorides (Bradbury and Crawford, 1977).

Finally, comparison of the results of the two series of assays indicates that comparable -NH_2 contents could be obtained using different combinations of the reaction parameters. For example, equal values of -NH_2 contents were obtained using either 5 min reaction with 2.5% TC (-NH_2 content: 98 μmol/g) or 30 min reaction with 1% TC (-NH_2 content: 99 μmol/g). However, this does not imply that the other functional groups of HSA were acylated to the same extent. In fact, microcapsules prepared with 2.5% TC (and 5 min reaction time) were found to belong to the type I group, whereas those obtained with 1% TC (and 30 min reaction time) exhibited a type II morphology with larger diameters and smooth membranes, thereby suggesting other structural differences (Lévy et al., unpublished results).

Conclusion

In our previous studies of HSA microcapsules, we showed that increasing polycondensation pH resulted in a decrease in microcapsule free amino groups paralleling the progressive acylation of hydroxy and carboxy groups. These structural changes were found to be very progressive. Accordingly, loosely cross-linked microcapsules could be prepared using pH values between 5.9 and 8. From a threshold value of 9, highly acylated membranes were prepared, corresponding to a marked decrease in microcapsule size to under 15 μm.

In this study, which was entirely conducted at pH 9.8, we compared the influence of reaction time and TC concentration on the acylation of HSA amino groups in microcapsules. As expected, increasing each parameter similarly resulted in a decrease in microcapsule -NH_2 content. However, no slow progression could be noted at this pH, an important acylation of amino groups being observed even for short reaction times or low TC concentrations. Accordingly, highly cross-linked (type I) microcapsules with small diameters (< 15 μm) were obtained under most conditions, while loosely cross-linked (type II) microcapsules with large diameters (30–40 μm) could only be observed using a 2 min reaction time or a 1% TC concentration. This observation confirms the determining role of reaction pH in acylation of protein functional groups. Considering variations of reaction time, it should be stressed that the results of our parallel study conducted using FT-IR spectroscopy are in good agreement with these observations. In fact, deeply modified spectra were recorded after 5 min, indicating high contents in esters and anhydrides.

This study then provided additional evidence for the rapid acylation of HSA functional groups in microcapsules prepared at a high reaction pH.

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