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Determination of free amino group content of serum albumin microcapsules using trinitrobenzenesulfonic acid: effect of variations in polycondensation pH

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Summary

Microcapsules were prepared from human serum albumin (HSA) through an interfacial cross-linking process using terephthaloyl chloride at various pH values (5.9–11). Determination of free amino groups was performed on lyophilized microcapsules by means of a back titration method using trinitrobenzenesulfonic acid (TNBS). Increasing reaction pH was shown to result in a progressive decrease of microcapsule free amino groups. Moreover, two groups of microcapsules could be distinguished with respect to their $-NH_2$ content: those obtained at low pH values (8 and < 8) which contained more than 400 μmol/g dry weight, and those prepared from pH 9, whose $-NH_2$ content did not exceed 110 μmol/g dry weight. These results were compared with previous findings concerning the involvement of hydroxy and carboxy groups of HSA microcapsules as a function of pH, that had been obtained from FT-IR spectroscopic studies.

Introduction

This study deals with microcapsules prepared from human serum albumin through an interfacial cross-linking process using terephthaloyl chloride (Lévy et al., 1982). In this process, several functional groups of the protein are involved in the formation of the membrane, as we have demonstrated in a study of HSA microcapsules using Fourier transform infrared (FT-IR) spectroscopy (Lévy et al., 1991). Besides amino groups (ϵ-amino groups of lysine and N-terminal amino groups) which are known to be readily acylated to form amide bonds, hydroxy and carboxy groups are also acylated. Actually, increasing reaction pH was shown to induce a progressive formation of ester and anhydride bonds in the membrane. However, it was not possible to evaluate the

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formation of amides by FT-IR spectroscopy, because these linkages could not be distinguished in the amide bands of HSA.

A chemical method had then to be used in order to evaluate the extent of acylation of amino groups in the modified protein. Procedures based on total hydrolysis of the constitutive protein and evaluation of unmodified lysine (Lee et al., 1981) were excluded, as amide bonds of acylated HSA would not survive the hydrolysis treatment. It was then decided to obtain this information indirectly by determining the amount of unreacted amino groups in the microcapsule membrane. A method has been described by Townes et al. (1989) for determination of surface $-NH_2$ groups of microparticles. The method is based on a coupling reaction with ^{14}C-labelled sodium acetate using a carbodiimide (EDCI) as the condensing agent and counting the samples in a liquid scintillation counter. It should be noted that hydroxy groups of the protein are also likely to condense with the activated carboxy group of acetate. Otherwise, this procedure is assumed to determine only accessible $-NH_2$ groups of the particles, due to steric reasons. However, it should be emphasized that the determination solely of the amount of unmasked functional groups, far from being a drawback, is of interest, as long as these groups are those which are involved in various properties of microparticles such as hydrophilicity, mucoadhesiveness and sensitivity to proteases. Moreover, only these accessible groups are concerned when amino groups of the microparticle surface are expected to combine with various linking agents for selective targeting or other purposes.

Keeping this observation in mind and looking for a simple procedure to determine free amino groups, we chose a method using 2,4,6-trinitrobenzenesulfonic acid (TNBS). In fact, this reagent has been found to react specifically and under mild conditions with the free primary amino groups to give trinitrophenyl (TNP) derivatives (Fig. 1), which can be determined spectrophotometrically (Satake et al., 1960).

The reaction has further been applied to various soluble compounds including amines, amino acids, peptides and proteins (Okuyama and Satake, 1960; Habeeb, 1966). A modification of the

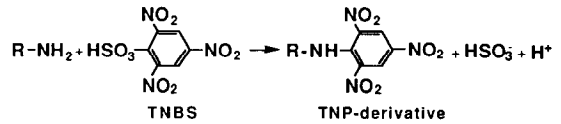

Fig. 1. Reaction of TNBS with amino compounds.

method has been described by Wand and Rudel (1978) allowing the determination of amino groups in insoluble materials. The procedure consists of the incubation of the material with an excess of TNBS and back titration of the unreacted amount of the reagent. This is achieved by adding valine to the filtered supernatant and performing a spectrophotometric determination of the resulting valine-TNP derivative. To our knowledge, this method has never been applied to microparticles. The purpose of the present study was to adapt the procedure to serum albumin microcapsules and to use the method for determination of microcapsule $-NH_2$ content as a function of cross-linking pH.

Batches of microcapsules were prepared at various pH values. The amounts of free amino groups were determined and compared. The results of the series of experiments were further compared with the conclusions of the FT-IR study and with the corresponding microcapsule size.

Materials and Methods

Materials

Microcapsules were prepared using lyophilized HSA purchased from CTS (Strasbourg, France). Terephthaloyl chloride was supplied by Janssen Chimica (France). Cyclohexane, chloroform and ethanol (Prolabo, France) were of analytical grade. The surfactants were sorbitane trioleate and polysorbate (Seppic-Montanoir, France). Determination of amino groups was performed using a TNBS solution containing 4 μmol/ml. This solution was prepared extemporaneously from a commercial 5% (w/v) aqueous solution (Sigma, France), by dilution with a 0.2 M borate buffer pH 8. DL-Valine (Aldrich Chimie, France) was

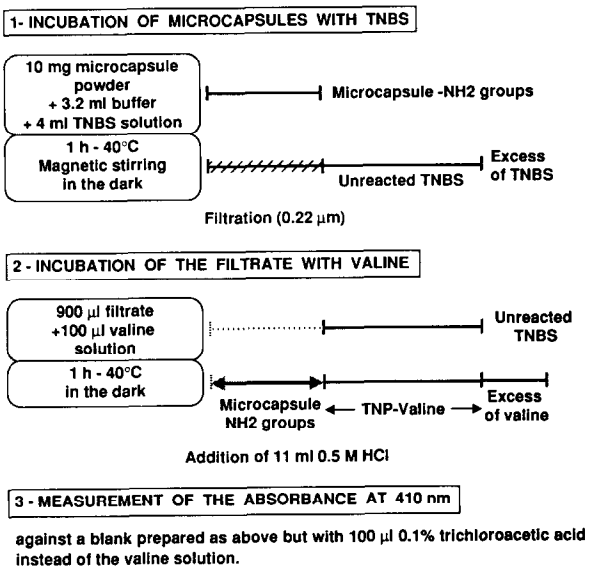

Fig. 2. Procedure for the determination of microcapsule -NH_2 groups using TNBS.

used as a 40 μmol/ml solution in 1% trichloroacetic acid.

Methods

Preparation of the microcapsules

Microcapsules were prepared using the interfacial cross-linking method under conditions which have been described previously (Lévy et al., 1991). Briefly, a 20% (w/v) HSA solution was prepared using either a carbonate buffer pH 9.8, or other buffers with different pH values (phosphate buffers pH 5.9, 6.8, 7.4 and 8 and carbonate buffers pH 9 and 11). This solution was emulsified in a chloroform:cyclohexane (1:4, v/v) mixture. Then, a 2.5% (w/v) solution of terephthaloyl chloride in the organic phase was added to the emulsion. After 30 min stirring, the microcapsules were washed, resuspended in water and lyophilized.

Determination of microcapsule free amino groups (Fig. 2)

In the first step, a sample of 10 mg of lyophilized microcapsule powder was moistened in a test tube with 3.2 ml of borate buffer pH 8. After 5 min magnetic stirring, 4 ml of TNBS solution was added. The test tube was incubated at 40°C in the dark with magnetic stirring for 1 h. The suspension was then filtered on a 0.22 μm filter. In the second step, 900 μl of the filtrate was introduced into a test tube and supplemented with 100 μl of the valine solution. The mixture was allowed to react at 40°C for 1 h in the dark. The reaction medium was then diluted with 11 ml of 0.5 M HCl, acidification resulting in stable-coloured TNP derivatives (Satake et al., 1960). The absorbance of the solution was measured at 410 nm against a blank treated as above but prepared with 100 μl of 1% trichloroacetic acid instead of the valine solution. A test tube was also prepared without microcapsules as a control in each series of assays.

The excess of TNBS was calculated from a calibration graph established with several dilutions of TNBS after reaction with valine: 0.9 ml of TNBS solution containing 0.5–2 μmol in borate buffer was introduced into test tubes. These test tubes were incubated at 40°C for 1 h in the dark. They were then supplemented with 0.1 ml of a 40 μmol/ml solution of valine in 1% trichloroacetic acid, incubated for a further 1 h in the dark and finally diluted with 11 ml 0.5 M HCl. Absorbance of all test tubes was measured against blanks treated as the test tubes and prepared for each TNBS dilution replacing the valine solution by 1% trichloroacetic acid. These precautions concerning the blanks make it possible to eliminate interference due to the hydrolytic product formed from TNBS, namely picric acid, and thus to obtain a linear relationship between absorbance and concentration.

The -NH_2 content of microcapsules was obtained by difference with the initial amount of TNBS. Mean values were calculated from four determinations: two samples of microcapsules were analyzed per batch and two batches of microcapsules were examined for each reaction pH value.

Results and Discussion

Applicability of the TNBS method to microcapsules

The TNBS method was successfully applied to cross-linked HSA microcapsules, which exhibited

a characteristic orange colour after 1 h incubation with the reagent. Microcapsules did not undergo degradation during the reaction and could then be readily separated, giving clear filtrates.

Table 1 displays the $-NH_2$ contents of microcapsules prepared at different pH values. It can be observed that the method is sensitive and provides reproducible results. Only slight variations were observed within each series of experiments. It should be pointed out that determinations were performed using dispersions of an insoluble material in the TNBS solution. Variable amounts of aggregates were then likely to remain in the reaction medium thereby reducing the number of accessible amino groups.

The highest value obtained in this study was 462.5 μmol $-NH_2$ per g microcapsule dry weight. Considering pure HSA, it can be calculated that the protein contains a maximal value of 855 μmol $-NH_2$/g, on the basis of 58 lysine ϵ-amino groups and one terminal α-amino group per molecule and a molecular weight of 69 000 (Peters, 1975). If we ignore the small amount of acylating agent which is present in the membrane and which should be deducted from the microcapsule weight, the decrease in microcapsule $-NH_2$ groups as compared with pure HSA may be attributed both to the acylation by terephthaloyl chloride and also to a partial reaction with TNBS. Actually, according to literature data (Goldfarb, 1966; Kakade and Liener, 1969), TNBS reacts rapidly with primary amino groups of proteins in solution, i.e., lysine ϵ-amino groups and N-terminal amino groups, but only partially, because a part of the amino groups is buried in the molecule or masked by interaction with proximal groups of the protein.

In this study, the TNBS method was applied to an insoluble cross-linked protein. The conditions used for the preparation of HSA microcapsules are assumed to induce some degree of unfolding of native HSA and thus to result in the exposure of buried residues. In fact, in an emulsion system, proteins tend to form an interfacial film which brings about conformational changes in the native molecule ('surface denaturation'; Cumper and Alexander, 1950). Otherwise, alkalinization is also known to favour denaturation of proteins, as is the case with HSA when increasing the pH from 7 to 9 ('N-B transition'; Leonard et al., 1963). Finally, acylation itself may have an additional unfolding effect on the protein by modifying the electric charges (Chang and Sun, 1978). The combination of these factors is then likely to bring to the periphery more lysine residues of HSA during the preparation of microcapsules. Nevertheless, the reaction of microcapsules with TNBS is presumably incomplete due to the above-discussed steric reasons.

Free amino group content as a function of cross-linking pH

As expected, increasing reaction pH was shown to result in a progressive decrease of free amino groups: the $-NH_2$ content was reduced by a factor of almost 10 when the pH increased from 5.9 to 11, alkalinization promoting acylation reactions (Table 1).

It was also observed that the disappearance of $-NH_2$ groups was especially marked from pH 9.

TABLE 1

Microcapsule $-NH_2$ content as a function of cross-linking pH

	Reaction pH													
	5.9		6.8		7.4		8		9		9.8		11	
Batch:	1	2	3	4	5	6	7	8	9	10	11	12	13	14
$-NH_2$ content	472	464	418	440	437	424	432	395	112	108	59	48	40	48
(μmol/g)	458	456	422	413	429	436	432	389	112	104	56	77	48	64
Mean value	462.5		423		431.5		412		109		60		50	

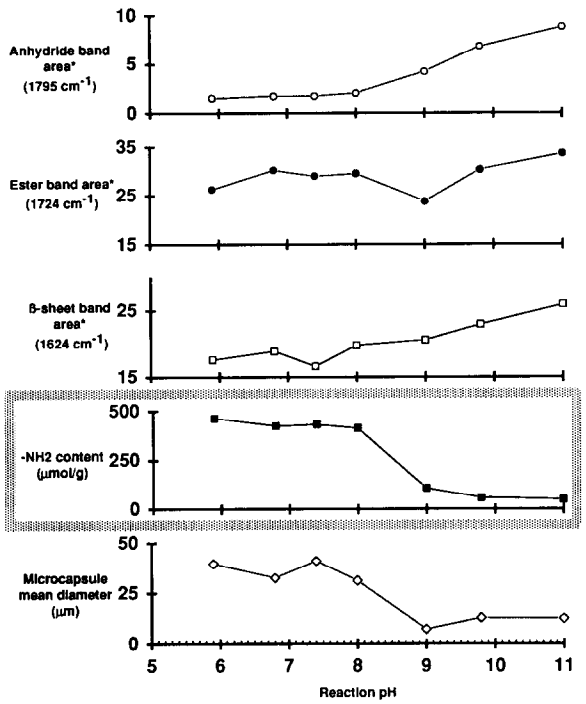

Fig. 3. Influence of reaction pH on the involvement of functional groups of HSA in microcapsule membrane and concurrent changes in microcapsule mean size. * From FT-IR spectroscopic studies (Lévy et al., 1991): arbitrary units.

This may indicate an additional influence of conformational changes of the protein leading to exposure of buried acylable groups, as we previously suggested (Lévy et al. 1991).

Accordingly, two groups of microcapsules could be distinguished with respect to their $-NH_2$ content: those obtained at low pH values (8 and < 8) which contained more than 400 μmol/g dry weight, and those prepared from pH 9, whose $-NH_2$ content did not exceed 110 μmol/g dry weight.

Comparison with the results of FT-IR spectrometric studies (Lévy et al., 1991)

The present results are in good agreement with our previous findings concerning the progressive acylation of hydroxy and carboxylate groups with increasing pH values as shown in Fig. 3. This effect is illustrated by an increase in the corresponding ester and anhydride band areas and by a concurrent increase in the β-sheet content which we had attributed to an evolution towards a more ordered structure.

Moreover, it had been shown that highly cross-linked microcapsules (typically microcapsules prepared at pH 9.8) exhibited a small size (< 15 μm) and a rough surface, while loosely cross-linked microcapsules displayed larger diameters (30–40 μm) and smooth membranes. The curves in Fig. 3 show that the changes in microcapsule size parallel the free amino group contents, with a marked decrease in size from pH 9 corresponding to a significant degree of acylation of amino groups.

Otherwise, it can be observed that, unlike anhydrides and amides, ester band areas exhibited a fall at pH 9 before rising again at pH 9.8 and 11. This unexpected phenomenon suggests the involvement of a concurrent reaction related to alkaline pH. Actually, it is well known that transacylation reactions may occur under alkaline conditions resulting in O-to-N acyl group transfer and in the formation of amides from esters. Transacylation might thus be involved here and participate in the marked fall in residual free amino groups observed at pH 9. The greater amounts of esters observed at pH 9.8 and 11 would then be accounted for by a more rapid acylation of HSA amino groups preventing acyl groups from being transferred from hydroxy groups to $-NH_2$ groups.

Finally, a careful examination of the variations in functional groups of loosely cross-linked microcapsules (pH 5.9–8) shows other unexpected irregularities (Fig. 3). Microcapsules prepared at pH 7.4 exhibited a higher $-NH_2$ content as compared with those obtained at pH 6.8. Likewise, the β-sheet and ester band areas decreased as compared with the values obtained at pH 6.8 (the areas of the anhydride bands were equal for the two pH values). In the search for a correlation with the change in microcapsule size, we could actually observe that microcapsules prepared at pH 7.4 exhibited larger diameters (mean size: 41 μm) than those prepared at pH 6.8 (mean size: 32.5 μm). The consistency of these observations strongly suggests that the physiological pH value represents a particular case corresponding to the

minimal denaturation of the protein and thereby the minimal exposure of acylable groups.

Conclusion

The TNBS method has been successfully applied to cross-linked HSA microcapsules and provided useful information on the involvement of amino groups of the protein in the membrane. The results are in good agreement with our previous findings concerning the involvement of hydroxy and carboxy groups in the walls as a function of pH.

Increasing the reaction pH resulted in a decrease of microcapsule $-NH_2$ content, while greater amounts of esters and anhydrides were shown to be formed from hydroxy and carboxylate groups, respectively. Moreover, acylation of amino groups was most pronounced from pH 9, corresponding to a significant decrease of microcapsule mean size.

The present study thus confirms the determining influence of polycondensation pH on interfacial cross-linking of proteins. It thus appears that the TNBS method might constitute a useful and simple method for measuring the available lysine content of protein microparticles.

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