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In vitro degradation of serum albumin microcapsules: effect of process variables

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Abstract

Microcapsules were prepared by cross-linking of human serum albumin (HSA) with terephthaloyl chloride (TC). Reaction pH was increased from 5.9 to 11, using 2.5% TC and 30 min reaction time. Then, in two series of assays conducted at pH 9.8, TC concentrations were varied from 0.5 to 5% (with 30 min reaction time), and reaction time was increased from 2 to 60 min (with 2.5% TC). Finally, two series of experiments were performed at pH 7.4 and 8, respectively, combining 2.5 and 5% TC with 30 and 60 min reaction time. Microcapsule degradation in pepsin (pH 1.2), and in trypsin (pH 7.5) was studied by microscopic examination. In the pH series, microcapsules prepared at pH ≤ 8 were degraded by pepsin within 50–70 min, while those obtained at pH ≥ 9 were resistant. Solubilisation time in trypsin was 3–10 min for pH ≤ 8, and 20–40 min for pH ≥ 9. At pH 9.8, resistance to pepsin was observed whatever the reaction time and the TC concentration, while solubilisation time in trypsin was 15–30 min. At pH 7.4 or 8, microcapsules were degraded in pepsin (50–70 min) and in trypsin (8–15 min). The results demonstrate the determining influence of reaction pH on microcapsule degradation. Besides, a correlation was found between microcapsule sensitivity to pepsin and high–NH_2 contents found in previous studies. © 1997 Elsevier Science B.V.

Keywords: Microcapsule; Human serum albumin; Interfacial cross-linking; Terephthaloyl chloride; Pepsin; Trypsin

1. Introduction

Serum albumin microparticles have been widely studied as parenteral drug delivery systems. Various methods have been reported for the preparation of microspheres, mainly based on heat-denaturation and chemical hardening using cross-linking agents (Gupta and Hung, 1989a,b). The requirement of biodegradability prompted several groups to investigate the effect of variations in the process variables on microsphere in vitro degradation by proteases. For example, raising the concentration of glutaraldehyde has been shown to result in a decreased sensitivity of

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the microspheres to chymotrypsin (Lee et al., 1981) and to trypsin (Willmott et al., 1985; Magee et al., 1995).

The purpose of this work was to investigate the enzymatic degradation properties of microcapsules prepared by interfacial cross-linking of human serum albumin (HSA) with terephthaloyl chloride (TC), as a function of several reaction parameters. The aim was to establish correlations between the sensitivity of microcapsules to proteases and the structural changes in HSA that we had previously shown using Fourier transform infrared (FT-IR) spectroscopy on the one hand (Lévy et al., 1991, 1994, 1995) and determination of the free amino group content of microcapsules with trinitrobenzene sulfonic acid (TNBS), on the other hand (Andry et al., 1996; Edwards-Lévy et al., 1993, 1994).

As a matter of fact, we have reported previously that increasing the reaction pH from 5.9 to 11 resulted in the progressive acylation of the various functional groups of HSA, namely amino groups (Edwards-Lévy et al., 1993), hydroxy groups and carboxylates (Lévy et al., 1991), forming amides, esters and anhydrides, respectively. These changes were most pronounced from pH 9 and were accompanied by a decrease in microcapsule size and by modification of the membrane surface, made rough (transition from 'type II microcapsules' to 'type I microcapsules'). We have also shown that varying TC concentration or reaction time at pH 9.8 did not result in important structural changes. In the two series of assays, microcapsule -NH_2 content was low and did not exceed 154 μmol/g (Edwards-Lévy et al., 1994), while an important acylation of hydroxy and carboxy groups was observed in most conditions except for a 2 min reaction time (Lévy et al., 1994) or a $\leq 1\%$ TC concentration (Lévy et al., 1995), for which microcapsules exhibited a large size and a smooth membrane (type II microcapsules).

Finally, studies were conducted at pH 7.4 and 8, using several combinations of TC concentration and reaction time. High values of $-NH_2$ content (>330 μmol/g) were found in all cases (Andry et al., 1996), associated with type II morphological characteristics (Andry and Lévy, unpublished data).

In this work, several series of experiments were carried out, varying first reaction pH, then TC concentration and reaction time at pH 9.8 and finally varying both TC concentration and reaction time at pH 7.4 and 8, respectively. Microcapsule in vitro degradation properties were studied using pepsin and trypsin. The solubilisation time was determined by microscopic examination. The results were compared to those of our previous studies.

2. Materials and methods

2.1. Materials

The HSA was supplied by CTS (Strasbourg, France). TC was purchased from Janssen Chimica, France. Cyclohexane and chloroform (Osi, France) were of analytical grade. The surfactants were sorbitan trioleate and polysorbate (Seppic, France). Pepsin (from porcine stomach mucosa) and trypsin (from porcine pancreas) were supplied by Sigma.

2.2. Preparation of the microcapsules (Lévy et al., 1991).

In the standard procedure, a 20% (w/v) HSA solution was prepared using a carbonate buffer pH 9.8. The solution (3 ml) was dispersed for 5 min at room temperature in 15 ml of a chloroform: cyclohexane (1:4, v/v) mixture containing 5% (v/v) sorbitan trioleate. Then, 20 ml of a 2.5% (w/v) solution of TC in the organic phase were added to the emulsion and stirring was continued for 30 min. The resulting microcapsules were separated, washed and lyophilized.

Variations were introduced in the standard procedure. The first series of experiments was conducted varying the reaction pH from 5.9 to 11. The following series of experiments carried out at pH 9.8, 7.4 and 8, respectively, varying TC concentration and reaction time.

Table 1
Microcapsule enzymatic degradation as a function of reaction pH correlation with -NH_2 content

Reaction pH	5.9	6.8	7.4	8	9	9.8	11
Solubilisation time in pepsin (min)	50–60	50–60	50–60	60–70	>20 h	>20 h	>20 h
Solubilisation time in trypsin (min)	3–6	3–6	8–10	8–10	20–25	20–25	35–40
NH_2 content[a] (μmol/g)	462.5	423	431.5	412	109	60	50

TC concentration, 2.5%; reaction time, 30 min.
[a] From a previous study (Edwards-Lévy et al., 1993).

2.3. Microcapsule enzymatic degradation

A solution of pepsin pH 1.2 was prepared according to U.S.P. XXI. Trypsin was used as a 0.25% solution in a phosphate buffer pH 7.5.

The enzymatic degradation assay was conducted as follows: a sample of 25 mg lyophilized microcapsules was rehydrated in a test tube with 1 ml distilled water and then dispersed in 7.5 ml of the enzymatic medium and incubated at 37°C. The solubilisation time was evaluated by microscopic examination and was defined as the time for disappearance of all microcapsules. All assays were triplicated. The results were expressed either by a single value, when equal times were found in the three assays, or by two values corresponding to the minimum and the maximum.

3. Results and discussion

3.1. Influence of reaction pH on microcapsule degradation properties

Table 1 presents the solubilisation time of microcapsules in pepsin and in trypsin as a function of reaction pH (with 2.5% TC and 30 min reaction time). For comparison, the table also shows microcapsule $-NH_2$ contents that have been determined in a previous study (Edwards-Lévy et al., 1993).

All batches prepared at pH ≤ 8 were degraded by pepsin within 50–70 min, while a complete resistance to pepsin was observed for microcapsules obtained at pH ≥ 9.

Then, loosely cross-linked type II microcapsules corresponding to high $-NH_2$ contents (>400 μmol/g dry weight) and low amounts of esters and anhydrides (Lévy et al., 1991), were also characterized by sensitivity to pepsin, while type I microcapsules having a low $-NH_2$ content (≤ 109 μmol/g) and a high content in esters and anhydrides were found to be resistant. It should be stressed that the same effect of raising the reaction pH on microcapsule degradation by pepsin has been observed previously with microcapsules prepared from gelatin with TC (Lévy et al., 1990).

Concerning trypsin, all batches were rapidly degraded, the solubilisation time increasing with growing pH values. In this case also the sensitivity to enzymatic degradation markedly decreased when raising the pH from 8 to 9 as shown by the solubilisation time which was prolonged from 8–10 min to 20–25 min.

3.2. Study at pH 9.8: influence of reaction time on microcapsule degradation properties

Table 2 shows the influence of reaction time (at pH 9.8, with 2.5% TC) on microcapsule degradation in pepsin and in trypsin.

First, it was observed that all microcapsule batches were resistant to pepsin. In trypsin, degradation time was comprised between 15 and 30 min in all cases.

Considering the results of the pH series, type II large-sized microcapsules obtained with 2 min reaction time were expected to be degraded by pepsin. However, these microcapsules had a low content in amino groups (≤ 154 μmol/g, Table 2), unlike the type II microcapsules of the pH series whose $-NH_2$ content was greater than 400 μmol/g. This difference might be involved in the observed resistance to pepsin.

Table 2
Microcapsules prepared at pH 9.8: enzymatic degradation as a function of reaction time (TC concentration, 2.5%)

Reaction time (min)	2	5	10	15	30	60
Solubilisation time in pepsin	>20 h	>20 h	>20 h	>20 h	>20 h	>20 h
Solubilisation time in trypsin (min)	15–20	15–20	15–20	20–25	20–25	25–30
$-NH_2$ content[a] (μmol/g)	154	98	90	73	60	75

[a] From a previous study (Edwards-Lévy et al., 1994).

3.3. Study at pH 9.8: influence of TC concentration on microcapsule degradation properties

Table 3 presents microcapsule solubilisation time as a function of TC concentration (at pH 9.8, with 30 min reaction time).

As observed with reaction time, variations in TC concentration did not result in significant changes in enzymatic degradation of microcapsules. All batches were also found resistant to pepsin, while they were degraded by trypsin within 15–30 min.

In this series, large-sized microcapsules with smooth membranes were obtained for TC concentrations $\leq 1\%$. Although having a type II morphology and low amounts of esters and anhydrides, these microcapsules exhibited a complete resistance to pepsin attributed to the low content in free amino groups (99 μmol/g for 1% TC, Edwards-Lévy et al., 1994).

3.4. Study at pH 7.4: influence of TC concentration and reaction time on microcapsule degradation properties

Table 4 presents the solubilisation time in pepsin and trypsin of microcapsules obtained at pH 7.4, combining 2.5 and 5% TC with 30 and 60 min reaction time.

All microcapsules were found to be degraded in pepsin within 50–60 min, and in trypsin within 8–12 min. Sensitivity to both proteases was associated with high amounts of free amino groups (≥ 338 μmol/g), no important variation in $-NH_2$ content being observed when prolonging reaction time and/or increasing TC concentration (Andry et al., 1996).

3.5. Study at pH 8: influence of TC concentration and reaction time on microcapsule degradation properties

The results of this series of assays are presented in Table 5. As observed in the preceding series, all microcapsule batches were digested by pepsin (solubilisation time: 60–70 min), and by trypsin (8–15 min). These microcapsules were also characterized by high $-NH_2$ contents (≥ 362 μmol/g, Table 5), as shown previously (Andry et al., 1996).

Taking these results together, it can be observed that all batches of serum albumin microcapsules were rapidly degraded by trypsin (solubilisation time ≤ 40 min), whatever the reaction conditions. The most significant variations in solubilisation time were found in the pH series with 3–6 min at pH 5.9 and 35–40 min at pH 11. Considering enzymatic specificity, it is known that the action of trypsin is restricted to peptide links involving the carboxylic groups of lysine and arginine, and that the hydrolytic action is slowed down when ϵ-amino groups of lysine residues are substituted, the splitting action being restricted to arginyl bonds (Desnuelle, 1960). Acylation of lysine ϵ-amino groups in microcapsules was then expected to delay the degradation in trypsin.

Considering pepsin, microcapsules were found to be either degraded within 50–70 min or resistant. In this study, microcapsule sensitivity to pepsin was associated with high -NH_2 contents (≥ 338 μmol/g), while resistance was observed for batches having low amounts of free amino groups.

The results the pH series of experiments show a correlation between the morphological and structural characteristics of microcapsules on

Table 3
Microcapsules prepared at pH 9.8: enzymatic degradation as a function of TC concentration (reaction time, 30 min)

TC concentration (%)	0.5	1	1.25	2.5	5
Solubilisation time in pepsin	>20 h	>20 h	>20 h	>20 h	>20 h
Solubilisation time in trypsin (min)	15	20	20	20–25	30
$-NH_2$ content[a] (μmol/g)	not determined	99	80	60	38

[a] From a previous study (Edwards-Lévy et al., 1994).

the one hand, and sensitivity to pepsin on the other hand. At pH ≤ 8, microcapsules had a type II morphology with a large size (31–41 μm) and a smooth membrane, which has been shown to correspond to loosely cross-linked membranes having a high content in free amino groups and low amounts of esters and anhydrides. These microcapsules were found to be degraded by pepsin. Inversely, microcapsules obtained at pH ≥ 9 had a type I morphology with a small size (7–13 μm) and a rough membrane, corresponding to highly cross-linked membranes having a low content in free amino groups and high amounts of esters and anhydrides. These microcapsules exhibited a complete resistance to pepsin.

Likewise, the results obtained at pH 7.4 and 8, varying the TC concentration and the reaction time, illustrate the correlation between type II morphological characteristics (Andry and Lévy, unpublished data) associated with high $-NH_2$ contents, and sensitivity to pepsin. As a matter of fact, at these pH values, it was not possible to observe a resistance to pepsin by prolonging the reaction time to 60 min and/or by increasing TC concentration to 5%, the microcapsule $-NH_2$ content remaining high under these conditions ($\geq$ 338 or ≥ 362 μmol/g at pH 7.4 and 8, respectively). FT-IR spectra would probably show low amounts of esters and anhydrides in the entire two series.

In the two series conducted at pH 9.8 varying reaction time and TC concentration, conditions were found for which the morphological type was not associated with the expected sensitivity to pepsin. As a matter of fact, microcapsules obtained with 2 min reaction time or 1% TC were shown to resist to pepsin, although they had a type II morphology and thus were expected to degrade in pepsin.

Considering structural characteristics, the expected lack of significant amounts of esters and anhydrides had been observed in both cases. However, the microcapsules exhibited low $-NH_2$ contents, thereby differing from the type II microcapsules of the pH series. This discrepancy has been attributed to an easier acylation of amino groups as compared with hydroxy and carboxylate groups. Accordingly, at pH 9.8 with 2 min reaction time or 1% TC, the involvement of the sole amino groups in the membrane would result in a looser network giving type II microcapsules. The resistance to pepsin would then be attributed to the masking effect of the numerous terephthaloyl groups attached to the amino groups of the membrane.

Considering enzymatic specificity, pepsin is known to act much more slowly than trypsin and to preferentially attack peptide bonds involving aromatic amino acids. The action is prevented by substitution on the aromatic ring (Dixon and Webb, 1965). Acylation of tyrosine hydroxy groups might then interfere in the resistance of highly cross-linked membranes having high amounts of esters. However, the involvement of additional unspecific factors was suggested by the results of a previous study that we conducted applying an hydroxylaminolysis treatment to highly cross-linked HSA microcapsules (reaction conditions: pH 9.8; 2.5% TC; 30 min reaction time). As a matter of fact, when these type I microcapsules were treated with alkaline hydroxylamine, a resistance to pepsin was still observed although ester and anhydride bonds were destroyed by the treatment providing hydroxamic groups. Amide bonds were the linkages involved in the membrane of the treated microcapsules, which were transformed into type II

Table 4
Microcapsules prepared at pH 7.4: enzymatic degradation as a function of TC concentration and reaction time

TC concentration (%)	2.5	2.5	5	5
Reaction time (min)	30	60	30	60
Solubilisation time in pepsin (min)	50–60	50–60	50–60	60
Solubilisation time in trypsin (min)	8–10	8–10	10–12	10–12
-NH_2 content[a] (μmol/g)	431.5	338	364	358

[a] From a previous study (Andry et al., 1996).

Table 5
Microcapsules prepared at pH 8: enzymatic degradation as a function of TC concentration and reaction time

TC concentration (%)	2.5	2.5	5	5
Reaction time (min)	30	60	30	60
Solubilisation time in pepsin (min)	60–70	70	70	70
Solubilisation time in trypsin (min)	8–10	13	13	15
–NH_2 content[a] (μmol/g)	412	426	374	362

[a] From a previous study (Andry et al., 1996).

microcapsules (with a larger size and a smooth membrane). An effect of steric hindrance by terephthaloyl groups in the membrane was then assumed to account for the observed resistance to pepsin (Hettler et al., 1994).

Accordingly, whatever the other reaction variables, microcapsules prepared at high pH values would exhibit a resistance to pepsin as a result of a rapid and important acylation of HSA amino groups.

In conclusion, this study demonstrates the determining influence of reaction pH on the degradation properties of cross-linked serum albumin microcapsules.

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