International Journal of Pharmaceutics, 69 (1991) R1–R4

ADONIS 037851739100117S

IJP 10026

Rapid Communication

Polyhydroxamic serum albumin microcapsules: preparation and chelating properties

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(Received 3 December 1990)
(Accepted 14 December 1990)

Key words: Chelating microcapsules; Iron; Human serum albumin; Interfacial cross-linking process; Hydroxamic acid

Summary

Polyhydroxamic serum albumin microcapsules were prepared through interfacial cross-linking with terephthaloyl chloride and further treatment of microcapsules with alkaline hydroxylamine. They exhibited iron-binding properties which were increased by esterification of the free carboxylic groups of the membrane with benzylic alcohol using a carbodiimide, prior to the hydroxylamine treatment.

The purpose of this communication is to present a novel type of chelating microcapsules prepared from human serum albumin (HSA) and bearing hydroxamic groups. They are prepared through an interfacial cross-linking process using terephthaloyl chloride (Lévy et al., 1982) followed by further treatment of the microcapsules with alkaline hydroxylamine. This work originates from a recent study that we performed on HSA microcapsules using FT-IR spectroscopy (Lévy et al., in press). We showed that increasing the polycondensation pH resulted in an increased involvement of ester and anhydride bonds in the microcapsule membrane and that treating the microcapsules with hydroxylamine under alkaline conditions induced the complete disappearance of the corresponding spectral alterations. This hydroxylaminolysis reaction is known to proceed more rapidly than the hydrolysis reaction and to result in the formation of hydroxamic groups (Hestrin, 1949). We therefore attempted to benefit from the hydroxamic groups that had been attached to the microcapsule membrane as a result of the transformation of terephthalic ester and anhydride groups. The well-known ability of polyhydroxamic compounds to sequester iron^{3+} (Varaprasad et al., 1986) prompted us to investigate the chelating properties of the microcapsules. Further assays were carried out in order to improve iron chelation by coupling benzyl alcohol to the free carboxylic groups of the membrane prior to hydroxylaminolysis.

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TABLE 1

Iron-binding capacity of HSA microcapsules: effect of treatment with NaOH or with alkaline hydroxylamine

Further treatment of microcapsules	Iron binding (μmol/g dry weight)						
	Batch 1		Batch 2		Batch 3		Mean value
–	0	0	0	0	0	0	0
3.5 M NaOH	18.1	16.6	16.6	15.4	15.4	15.4	16.2
1 M NH_2OH + 3.5 M NaOH	33.1	32.7	31.9	30.5	30.6	32.6	31.9
3 M NH_2OH + 3.5 M NaOH	33.6	33.4	33.0	35.3	36.4	35.5	34.5

In all experiments, HSA (Centre de Transfusion sanguine, Strasbourg, France) was used as a 20% (w/v) solution in a 0.45 M carbonate buffer, adjusted to pH 9.8 with HCl. Microcapsule batches were prepared using the following procedure: the HSA solution (12 ml) was emulsified in 60 ml of chloroform : cyclohexane (1 : 4, v/v) containing 5% (v/v) sorbitan trioleate (Seppic, France). After 5 min stirring (Heidolph stirrer, Prolabo, France; stirring speed: 2400 rpm), a 2.5% (w/v) solution of terephthaloyl chloride (Janssen Chimica, France) in the solvent mixture (80 ml) was added and stirring was continued for 30 min. The reaction medium was then diluted with 40 ml of organic phase. The microcapsules were centrifuged and washed successively with the solvent mixture, with a 2% (v/v) polysorbate solution in 95% ethanol, with 95% ethanol and with water.

In a first series of experiments, the obtained microcapsule aqueous suspension was divided into four parts (Table 1). One part was kept untreated as a control, and was congealed and lyophilized. An alkaline hydroxylamine treatment was applied to a second fraction, under the conditions used in our FT-IR spectroscopic studies: freshly washed microcapsules were centrifuged, resuspended in 20 ml distilled water and successively added with 4 ml of 1 M hydroxylamine hydrochloride (Prolabo, France) solution, and with 4 ml of 3.5 M NaOH. After 15 min magnetic stirring at room temperature, the microcapsules were rinsed with distilled water, until washing media pH became neutral. They were then congealed and lyophilized. A similar treatment was applied to a third fraction, using 3 M hydroxylamine. The last part was treated only with 3.5 M NaOH as a control. Two samples were taken in each lyophilized part for determination of iron chelation. This experiment was triplicated.

In the second series of assays, a batch of HSA microcapsules was prepared and washed with the solvent mixture. They were then resuspended in fresh solvent and the suspension was divided into 3 parts (Table 2). One part was kept ungrafted as a control and further washed with 2% polysorbate solution in 95% ethanol, with 95% ethanol and with water. After centrifugation, these microcapsules were soaked in alkaline 3 M hydroxylamine and subsequently washed and lyophilized. The two other parts were grafted with benzyl alcohol using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) as the condensing agent. This reaction was performed using triethyl-

TABLE 2

Iron-binding capacity of HSA microcapsules: effect of grafting with benzyl alcohol prior to treatment with alkaline 3 M hydroxylamine

Grafting with $C_6H_5CH_2OH$	Iron binding (μmol/g dry weight)						
	Batch 4		Batch 5		Batch 6		Mean value
–	37.2	37.7	30.5	29.1	31.5	31.4	32.9
8 mmol	48.0	48.0	49.8	49.0	44.2	45.3	47.4
24 mmol	42.2	44.5	47.8	48.0	42.2	45.2	45.0

amine (Sheehan et al., 1965), which is known to prevent the intermediary *O*-acylurea from reacting with another acidic group (Mikolajczyk and Kielbasinski, 1981) and is thus likely to favour the formation of esters. Freshly prepared microcapsules, after being washed with the solvent mixture, were suspended in 1 ml of dichloromethane. A solution of 4 mmol of EDCI (Janssen Chimica, France) in 8 ml of dichloromethane was then added. After 15 min stirring (900 rpm), 10 ml of a solution containing 8 or 24 mmol benzyl alcohol (Merck, France) and 4 mmol triethylamine (Janssen Chimica) in dichloromethane was added. Agitation was maintained for 2 h. The microcapsules were then centrifuged and washed with 95% ethanol and with water. They were finally treated with alkaline 3M hydroxylamine, rinsed and lyophilized as above. Two samples were taken in each lyophilized part for evaluation of the chelating properties. This experiment was triplicated.

Microcapsule morphology and size range were assessed by optical microscopy and scanning electron microscopy (SEM). The iron-binding properties were studied using a 140 μmol/l $FeCl_3$ solution. A sample of 25 mg lyophilized microcapsule powder was accurately weighed, placed in a beaker and 10 ml of the ferric solution was added. After 2 h magnetic stirring, the supernatant was filtered through a 0.22 μm filter and assayed for iron content using a colorimetric method based on the formation of a complex with Ferrozine^R. The measurement was performed at 570 nm by means of an automatic analysis system (BM Hitachi System 717, Boehringer Mannheim GmbH Diagnostica).

Microcapsules were shown to survive the treatments with alkaline 1 M or 3 M hydroxylamine as well as with 3.5 M NaOH, as seen by microscopic examination. Spherical transparent particles were observed with a mean size around 20 μm. After soaking in the ferric solution, all hydroxylamine-treated samples, especially the grafted ones, exhibited the orange-red colour characteristic of the hydroxamic acid-Fe complex (Winston, 1984). After washing with distilled water and lyophilization, they could be directly observed by SEM without metallization.

Table 1 displays the values of iron binding of HSA microcapsules depending on the further treatment used. Untreated microcapsules did not bind iron, while some fixation was observed with NaOH-treated microcapsules. This result is attributed to the hydrolysis of anhydride, ester and probably thioester groups, making carboxylic, hydroxy and thiol groups available for metal binding. However, the determining effect of the hydroxylamine treatment was demonstrated by the significant increase in iron binding observed with 1 M or 3 M hydroxylamine-treated samples. They showed comparable and reproducible chelating properties. Furthermore, as shown in Table 2, microcapsule iron binding could be improved by 45% through esterification of the free carboxylic groups with 8 mmol benzyl alcohol prior to the 3 M hydroxylamine treatment, due to the formation of an additional amount of hydroxamic acids. Raising the benzylic alcohol amount to 24 mmol did not further improve iron binding, which was shown to be highly reproducible.

In the present study, application of the hydroxylamine treatment to crosslinked HSA microcapsules proved to be an efficient means of yielding iron-binding microcapsules, especially after esterification of the carboxylic groups of the wall. As hydroxamic groups are known for their remarkable binding properties towards various metal ions (Coutts, 1967), polyhydroxamic HSA microcapsules appear as a new versatile tool, that might chelate various radionuclides, as well as Fe^{3+} or other paramagnetic elements for magnetic resonance imaging. Among the variety of means proposed for attaching metal-chelating groups to HSA microspheres (Wagner and Welch, 1979; Hnatowich and Schlegel, 1981), polyhydroxamic HSA microcapsules, which are prepared using a simple and rapid process, thus appear as a promising approach.

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