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Influence of formulation on the physicochemical properties of casein microparticles

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Abstract

Casein microparticles (CAS/MP) have a potential clinical use for targeting drugs. However, the use of organic solvents in their preparation is undesirable. This study was designed to investigate the influence of preparation procedures in aqueous media on the formulation and physicochemical properties of CAS/MP. The first stage involved the influence of the coacervating agents (lactic acid, succinic anhydride, succinic acid and tartaric acid). The second stage studied was the influence of the ionic strength and the third, the influence of adding a thickener, hydroxypropyl cellulose or hydroxypropyl methycellulose (HPC or HPMC), and a plasticizing agent (gelatin). Some physicochemical properties of CAS/MP were evaluated. While the infrared and the thermal analysis showed that all coacervating agents were appropriate for coacervation, the scanning electron microscopy studies showed that the external morphology of the particles was more homogeneous when lactic acid was used. Utilizing lactic acid as the coacervating agent, there was a trend effect of adding NaCl implying that the increasing of the ionic strength resulted in better stability. Finally, the addition of 0.1% HPC plus either 0.25 or 0.5% gelatin resulted in homogeneous formulations. In conclusion, the use of lactic acid plus 0.1% HPC and 0.25% gelatin results in biodegradable and homogeneous CAS/MP, presenting a potentially useful drug delivery system.

Keywords: Aqueous coacervation; Casein; Microencapsulation; Process variables; Protein microparticles

1. Introduction

There has been great interest in developing systems capable of controlling the rate of drug delivery, prolonging the duration of therapeutic effect and targeting the delivery of drug to a tissue (Chien, 1992; Gibaud et al., 1996).

One approach to produce sustained release of drugs is by the use of multiparticulate drug delivery systems. Many polymers have been used, such as cellulose derivatives, methacrylic–acrylic acid derivatives (Watts et al., 1994; Das and Das,

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1998), polylactic acid and related polymers (Ghaderi et al., 1996; Shiga et al., 1996), polysaccharides (Lim and Wan, 1998), proteins (Leo et al., 1997) and other materials. Some of these polymers and the microencapsulation procedure may offer hazards by contamination with organic solvents and intermediaries (Bodmeier and Wang, 1993). There arises the necessity to search for biodegradable materials that will be acceptable to the patient and the environment (Ishizaka et al., 1985).

Recently, casein microspheres have been investigated as carriers of cytotoxic drugs, since casein is more available than serum albumin (Chen et al., 1987; Latha et al., 1995). There has been a great interest in its use in preparing casein microspheres with good spherical structure and precise control of particle size (Jayakrishnan et al., 1994). Acylated proteins may be used for targeting protein drugs (Takakura et al., 1994).

The objectives of this study were two-fold. Firstly, to prepare casein microparticles (CAS/MP) using an organic solvent-free system. Secondly, to determine the influence of different preparation procedures on some physicochemical properties of coacervated CAS/MP such as: (1) thermal and spectroscopic profiles; (2) morphological properties; and (3) particle size and distribution which control drug release properties.

2. Materials and methods

2.1. Materials

Bovine casein (biological grade; Inlab®, SP, Brazil). Lactic acid, succinic acid, tartaric acid and succinic anhydride (analytical grade) (Sigma, USA). Biomedical grade hydroxypropyl cellulose (HPC) and hydroxypropyl methylcellulose (HPMC) were a gift from Klucel®-Aqualon. Carbopol 940 (B.F. Goodrich, OH). Lecithin (biological grade; Sambra® Brazil). Tween 80 (Sigma). Glutaraldehyde (Fluka Chemika, Switzerland), used as a cross-linking agent. Gelatin (microbiological grade; Farma & CIA) was used as plasticizer agent. All other reagents were analytical grade and were used as received.

2.2. Methods

2.2.1. Preparation of casein microparticles (CAS/MP)

CAS/MP were obtained by a simple coacervation method developed in aqueous medium using a 500-ml glass beaker equipped with a Diax 600 (Heidolph-Elektro GmbH & KG D-8420 Kelhem, Germany). The stirring speed was set constant at 8000 rpm at room temperature (~ 28°C).

Table 1 lists three different coacervation solutions used. Four different coacervating agents were used (lactic acid, tartaric acid, succinic acid or succinic anhydride) in formulation A. Lactic acid was used as the coacervating agent with 10 or 20 mM NaCl in order to investigate the influence of enhancing of ionic strength (formulation B). Finally, in formulation C, sodium lactate buffer 2 or 20 mM with different amounts of HPC or HPMC (0.1 or 0.2%, w/v) and gelatin (0.25 or 0.5%, w/w) were used to investigate if the use of thickener and plasticizing agents in buffered coacervating medium would influence the formation of CAS/MP.

Dispersions of casein (5 mg/ml) were prepared in water by the addition of 5 ml of 2.5 M NaOH and the pH was adjusted to 11.0. The casein dispersion was added to the coacervating solution (A, B or C), stirred for 2 h, then 3% of glutaraldehyde 50% (v/v) was added to the preparation and the agitation was continued for another hour. The CAS/MP were filtered and washed under vacuum using a mix of ethanol/water, and finally suspended in ethanol for drying.

Table 1
Casein dispersion coacervated by different methods

Formulations		
A	B	C
0.5% Lecithin (v/v)	0.5% Lecithin (v/v)	2 or 20 mM lactate buffer
0.1% Carbopol 940 (w/v)	0.9% Lactic acid (w/v)	0.1 or 0.2% HPMC or HPC (w/v)
0.9% Coacervating agent[a] (w/v)	10 or 20 mM NaCl	0.25 or 0.5% gelatin (w/w)

[a] Succinic anhydride, lactic, succinic and tartaric acids.

2.2.2. Drying technique

The CAS/MP ethanol suspensions were dried using a 'bench spouted bed drier' in accordance with Mathur and Epstein (1974), measuring 9 cm diameter, 25 cm in height, and 5 cm air entrance diameter, using glass beads, (mean diameter 2.6 mm), under constant air pressure and at 50°C.

The CAS/MP were dropped on the top of the bed at a flow rate of 8.5 ml min^{-1}.

2.2.3. Morphology of microparticles

The external morphology of CAS/MP was assessed by scanning electron microscopy (SEM), using a Cambridge 360 SEM instrument.

2.2.4. Size distribution

Particle sizes and distributions of dried CAS/MP were determined by sieving using a standard series of test sieves in the range of 25–710 μm.

The technique is made up of weighing the sieves individually before and after 30 min on a shaker table (120 rpm). The results were evaluated by a frequency distribution curve, where the number of particles lying within a certain size range is plotted against the size range or mean particle size (Martin, 1993), providing the percentage of particles in the range related to the total amount.

2.2.5. FT-IR spectroscopy

Fourier transform infrared (FT-IR) spectroscopy was used to investigate physicochemical interactions occurring in the CAS/MP. The samples used for spectral measurements were prepared according to the KBr technique.

2.2.5.1. Preparation of samples. Known amounts of CAS/MP and KBr were mixed and compressed to form a transparent disc under a pressure of 10 metric tons. The disc was placed in a holder and scanned directly in the spectrometer.

2.2.5.2. FT-IR spectra. A Nicolet-5-ZDX FT-IR spectrophotometer, over a scan range of 4000–500 cm^{-1} under 4 cm^{-1} resolution, was used at 28°C.

2.2.6. Thermal analysis

DSC analysis was carried out using a DSC-50 Shimadzu, where ~5-mg samples of CAS/MP were submitted to a constant heating rate of 10°C min^{-1} from 25 to 290°C using nitrogen as the sweeping gas (25 ml min^{-1}).

3. Results and discussion

3.1. Surface morphological analysis

The surface morphology of CAS/MP is shown in Fig. 1a (succinic anhydride), Fig. 1b (succinic acid), Fig. 1c (lactic acid) and Fig. 1d (tartaric acid). Although the CAS/MP coacervated with either succinic anhydride or succinic or tartaric acid showed a non-homogenous form and mainly particle aggregates, the morphology of CAS/MP coacervated with lactic acid showed a good spherical shape with a relatively smooth surface, and particles dispersed in a non-aggregated form.

The results from the SEM confirm the use of lactic acid as the coacervating agent of choice for CAS/MP formulations.

3.2. Size distribution

Fig. 2 shows frequency distribution plots (FDP) of samples prepared from CAS/MP in a medium containing 0.2% of HPMC as thickening agent and 0.5% of gelatin as plasticizing agent in known concentrations of sodium lactate buffer (pH 3.8). When 20 mM buffered medium was used, containing 34% of CAS/MP within the range, there was a better uniformity of size distribution: 180–355 μm. Based on these results, further systems were prepared in 20 mM buffered medium. The FT-IR spectroscopy and DSC data did not detect changes in protein structure.

Fig. 3 shows the influence of the use of increasing amounts of thickening agent, and it can be observed that the particle size and distribution of CAS/MP was not influenced significantly. However, the comparison between different thickening agents showed important differences in the uniformity of the particle size distribution of the CAS/MP. The use of 0.2% of HPC led to 47% of

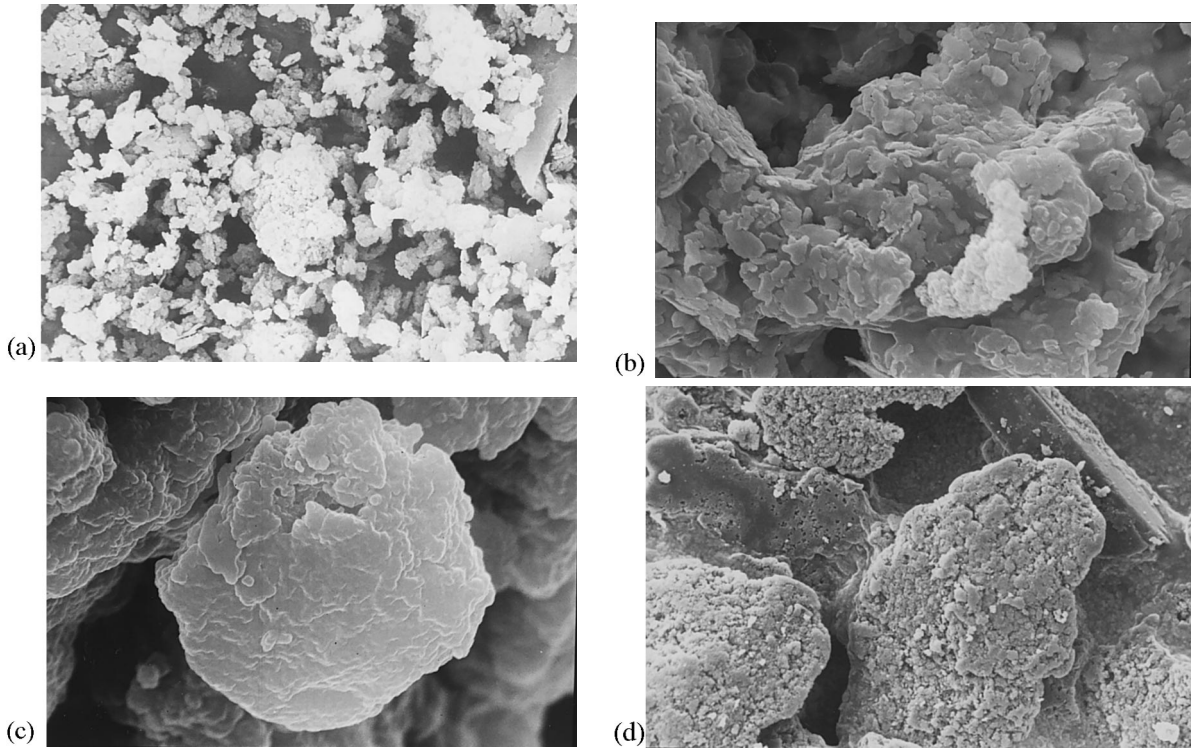

Fig. 1. (a) Photomicrograph of CAS/MP coacervated with succinic anhydride. (b) Photomicrograph of CAS/MP coacervated with succinic acid. (c) Photomicrograph of CAS/MP coacervated with lactic acid. (d) Photomicrograph of CAS/MP coacervated with tartaric acid.

particles being in the range of 180–355 μm, indicating better homogeneity of distribution when compared with the use of HPMC under the same conditions. Consequently, increased amount of less hydrophilic thickening agent, such as HPC, resulted in more homogeneous distribution of particles in the same size range.

Other results referring to the influence of gelatin on the amount of HPC required as thickener indicated that the presence of 0.25 or 0.5% of gelatin resulted in a suitable size distribution, suggesting that 0.1% HPC plus 0.25% gelatin is a suitable formulation for preparation of CAS/MP (data not shown).

3.3. Physicochemical properties

3.3.1. Infrared spectroscopy

The spectroscopic analysis of polymeric molecules, including proteins (Cooper and Knutson, 1995), is complicated by the complexity of molecular vibrations arising from numerous atoms of a molecule contributing to a particular vibration. In this work, infrared spectroscopy was used to investigate the occurrence of interactions among specific protein sites and coacervating agent, as well as cross-linking agent. Such interactions must be recognized through spectral changes from native polymer and the same polymer submitted to a microencapsulation procedure.

The secondary structure of proteins is derived from the conformation-sensitive amide bands, notably from the so-called amide I, II and III bands in the spectral region between 1700 and 1200 cm^{-1} (Levy et al., 1991; Surewicz et al., 1993). The other bands in this spectral region, due to side chain vibrations, provide additional information concerning the functional groups of proteins and their involvement in chemical bonds with acylating agents.

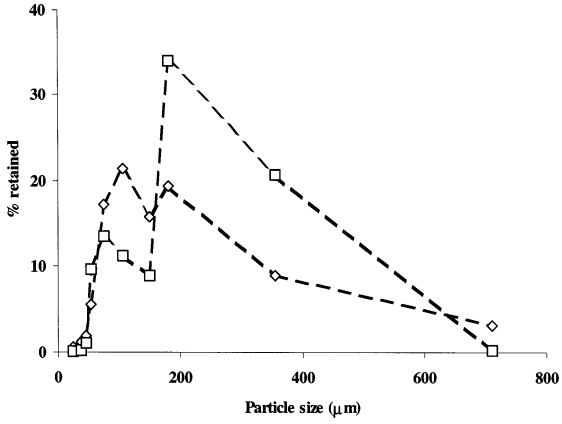

Fig. 2. Frequency distribution plot of CAS/MP prepared using variable concentrations of lactate buffer containing 0.2% HPMC plus 0.5% gelatin: (◇) 2 mM and (□) 20 mM.

While cross-linking of polysaccharides only involves hydroxyl groups in the formation of ester bonds, various functional groups of proteins may be involved in the reaction, namely free NH_2 and NH groups, alcoholic groups of hydroxyl amino acids, phenolic groups of tyrosine, and thiol groups of cysteine. Moreover, it may be assumed that the involvement of these various groups will vary qualitatively and quantitatively, according to the cross-linking conditions, thereby determining microparticle properties, such as biodegradability and interactions with entrapped drugs.

In general, the FT-IR results demonstrated some changes in specific regions in all spectra, i.e. Fig. 4A shows the IR spectra of casein and CAS/MP coacervated by succinic acid. Regions of 1650 cm^{-1} referred to C=O and C–N stretching from amide II and 1100 cm^{-1} due to C–O and C–N stretching in the intact protein, which were modified after the coacervation method used in this work. In addition, changes in the 1050–950 cm^{-1} region could correspond to side chain vibrations from protein (Levy et al., 1991), which even though they cannot be classified as a functional specific group, also indicate alteration of protein structure under investigation, permitting the suggestion that modifications in such chains can occur.

The peak in the 1725 cm^{-1} band is characteristic of C=O stretching vibrations, and may be presumably a consequence of the organic acid used to coacervate the casein molecules, i.e. a carboxylic or a dicarboxylic acid.

Fig. 4B shows that neither the addition of a plasticizing agent nor a thickening agent interfered with the functional groups of the CAS/MP. Furthermore, an increase in the ionic strength did not influence significantly the spectroscopic behavior, suggestive of no influence on protein structure.

In agreement with other workers (Burgess and Singh, 1993) who suggested that the pH decrease

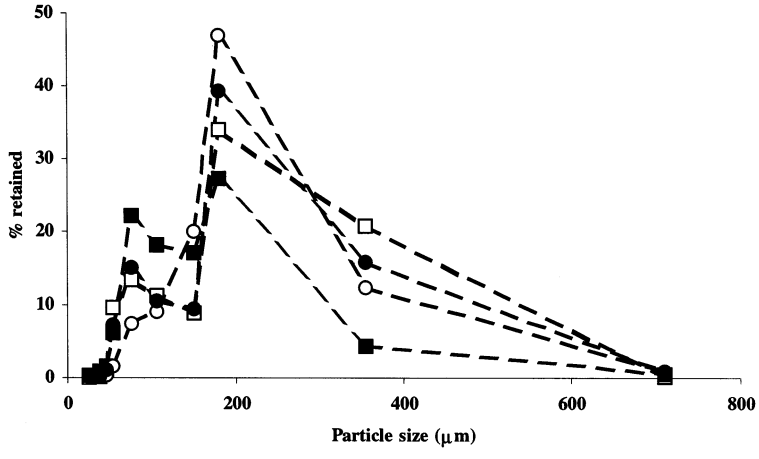

Fig. 3. Frequency distribution plot of CAS/MP prepared using 0.5% of gelatin and variable concentrations of HPMC and HPC: (□) 0.2% HPMC; (■) 0.1% HPMC; (○) 0.2% HPC; (●) 0.1% HPC.

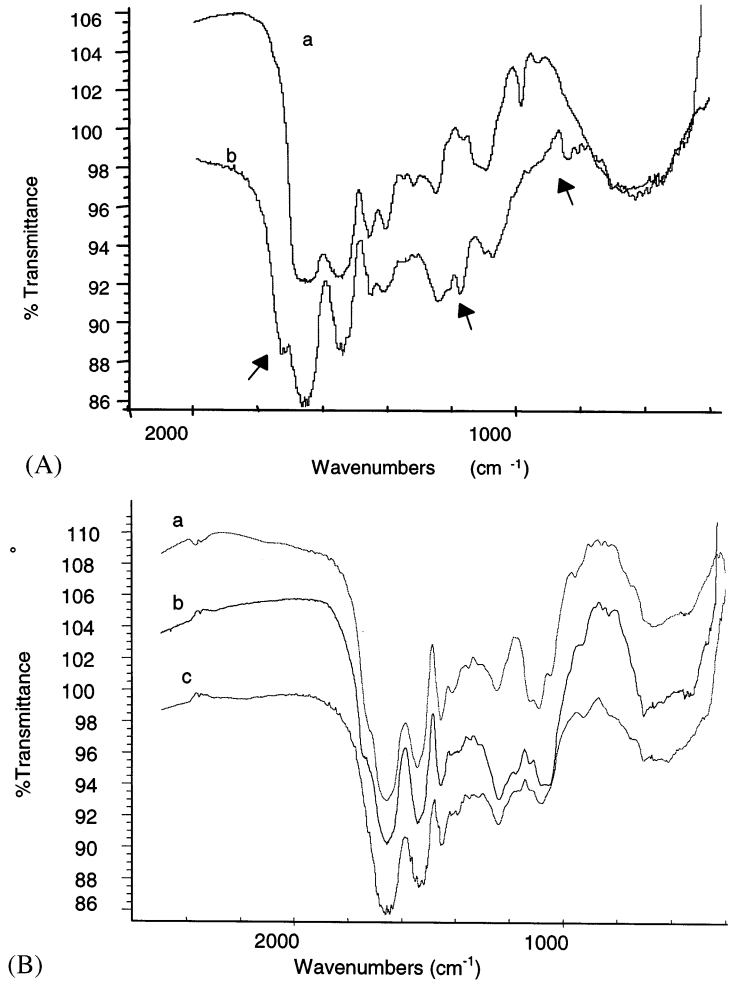

Fig. 4. (A) FT-IR spectra of casein (a) and CAS/MP coacervated by succinic acid (b). (B) FT-IR spectra of coacervated CAS/MP by: lactic acid (a); lactic acid containing 20 mM of NaCl (b); sodium lactate buffer 20 mM containing 0.5% of gelatin and 0.2% of HPC (c).

and the ionic strength change can be important in the coacervation process, we have seen through FT-IR spectroscopy that this process was independent of the acylating agent used (lactic acid, succinic anhydride, succinic acid or tartaric acid). Thus, such coacervation was a consequence of the acylation rather than the ionic strength alteration.

In addition, it is meaningful to comment that the acylation rate is dependent on the pH (Levy et al., 1991), becoming less at lower pH values. Levy et al. (1991) worked with cross-linked human serum albumin to prepare microcapsules, which were assessed by FT-IR. The most notable changes were found in three regions dominated by the amide I and III bands. The amide I band, located in the 1690–1600 cm^{-1} region, resulted from the C=O stretching vibration coupled to the in-plane N–H bending and C–N stretching modes. The amide III band generally falls within the 1300–1230 cm^{-1} interval mainly associated with the C–N stretching and in-plane N–H deformation modes of the peptide group. All the other bands are due to side chain vibrations.

3.3.2. Thermal analysis

Thermal analysis data confirm the results obtained by FT-IR analysis. It can be seen in Fig. 5 that there was an increase in the main transition from 90 to 110°C, characterized as an endothermic event. This change is probably related to dehydration of the coacervated protein formulation as compared to the native casein, showing that the original polymer undergoes physical or chemical changes after coacervation process corresponding with an increase of its enthalpy.

The coacervating agents used have induced new secondary transitions in the range of 190–210°C, which were not seen with casein (Fig. 5), suggesting a change in the original protein depending on the acylation agent used. Although the extent of low acylation due to the medium pH (3.8) is low it can be assumed that such a reaction accompanies the microencapsulation method used.

4. Conclusions

This work describes a method of preparing casein microparticles in aqueous medium by simple coacervation. Analysis of the microparticles (FT-IR and DSC) demonstrated that the coacervation was a consequence of the acylation rather than the alteration of the ionic strength of the aqueous medium. Nevertheless, the SEM analysis showed that the use of lactic acid as coacervating agent resulted in a good spherical shape with relatively smooth surface particles compared to the other coacervating agents used. The plasticizing and thickening agents used demonstrated that the sodium lactate-buffered 20 mM medium plus 0.1% of HPC plus 0.25% of gelatin as coacervating solution, result in more homogeneous CAS/MP, presenting a practical interest and application for delivering drugs in a less harmful formulation.

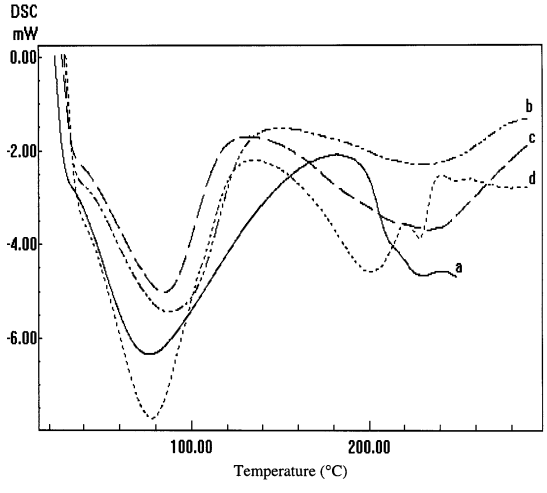

Fig. 5. DSC of casein (a) and CAS/MP prepared by different coacervating agents: lactic acid (b), succinic anhydride (c), tartaric acid (d).

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