Carboxymethyl starch/alginate microspheres containing diamine oxidase for intestinal targeting

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Abstract

The association of carboxymethyl starch (CMS) and alginate is proposed as a novel matrix for the entrapment of bioactive agents in microspheres affording their protection against gastrointestinal degradation. In this study, the enzyme diamine oxidase (DAO) from white pea (*Lathyrus sativus*) was immobilized by inclusion in microspheres formed by ionotropic gelation of CMS/alginate by complexation with Ca^{2+}. The association of CMS to alginate generated a more compact structure presenting a lesser porosity, thus decreasing the access of gastric fluid inside the microspheres and preventing the loss of entrapped enzyme. Moreover, the immobilized enzyme remained active and was able to oxidize the polyamine substrates even in the presence of degrading proteases of pancreatin. The inclusion yield in terms of entrapped protein was of about 82%–95%. The DAO entrapped in calcium CMS/alginate beads retained up to 70% of its initial activity in simulated gastric fluid (pH 2.0). In simulated intestinal fluid (pH 7.2) with pancreatin, an overall retention of 65% of activity for the immobilized DAO was observed over 24 H, whereas in similar conditions the free enzyme was totally inactivated. Our project proposes the vegetal DAO as an antihistaminic agent orally administered to treat food histaminosis and colon inflammation. © 2015 The Authors. *Biotechnology and Applied Biochemistry* published by Wiley Periodicals, Inc. on behalf of the International Union of Biochemistry and Molecular Biology, Inc. Volume 63, Number 3, Pages 344–353, 2016

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Keywords: *antihistaminic diamine oxidase, carboxymethyl starch/alginate microspheres, colon delivery, Crohn's disease and ulcerative colitis, gastrointestinal-resistant therapeutic enzymes, inflammatory bowel diseases*

1. Introduction

Drug carrier systems employ biodegradable and biocompatible polymeric excipients to formulate tablets, capsules, or microspheres. For compliance of patients, especially for children, elders, or postsurgery subjects, there is an interest for smaller and soft dosage forms. Microspheres are easy to administrate orally and various bioactive agents and biopolymers such as poly(lactic-glycolic) acid [1], chitosan [2], and alginate [3] have been used as polymeric matrices for active principle formulation. Encapsulation with poly(lactic-glycolic) acid microspheres requires a relatively complex processing often involving solvents, which could lead to the denaturation of bioactive agents [1]. Chitosan would liberate only a part of bioactive agents in the colon tract because of its limited degradation by bacterial enzymes [4]. Alginate, a natural polysaccharide from seaweed that consists of β-D-mannuronic and α-L-guluronic acid residues with β-1,4 glycosidic links [5], is largely used as matrix to produce microspheres in controlled delivery because of its biocompatibility, low toxicity, and good mechanical properties [6, 7]. Gel beads may be formed by spreading a solution of sodium alginate into a divalent Ca^{2+} cation solution.

Abbreviations: *CMS, carboxymethyl starch; DAO, diamine oxidase; FTIR, Fourier transform infrared; IBD, inflammatory bowel disease; SEM, scanning electron microscopy; SGF, simulated gastric fluid; SIF, simulated intestinal fluid.*

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The mechanical properties of microspheres depend on the composition, sequential structure, and molecular size of the polymers as well as on the concentration of alginate and the cation solution [8, 9]. There is a direct dependence between the guluronic–mannuronic acid distribution, the mechanical strength of alginate gel, and the porosity of the gel network. Gels made from an alginate with a high α-L-guluronic acid content result in a higher porosity compared to gels made from alginate with a lesser α-L-guluronic acid content [10]. Furthermore, high guluronate alginate gels are rigid and brittle, whereas gels produced from a low guluronate alginate are more elastic [8]. Previous studies showed that the entrapment in alginate microbeads or films offered good protection and satisfactory delivery for organic compounds [11], bacteria [12], and enzymes [13]. However, a certain porosity of microspheres can limit the stability of the entrapped bioactive agents [12]. The association of alginate with other polymers can improve the properties of the matrix. For example, chitosan has been used as a coating for alginate microspheres via a polyelectrolyte association to delay the diffusion rate of the encapsulated substances [14] or as an additive that modifies the structure of the beads [15]. In this context, we have hypothesized that the association of a hydrogel material with alginate would reduce the porosity and thus delay the diffusion of gastric acidity into the microspheres, particularly in conditions of protonation of carboxylic groups [16], which will make the microbeads more compact.

Carboxymethyl starch (CMS) is a biodegradable polymer that has been proposed as an excipient for controlled release of small molecules and bioactive agents in oral solid monolithic tablets dosage forms [16]. CMS is an anionic starch modified by treatment with monochloroacetic acid in order to introduce carboxylic groups by etherification. As powder, CMS hydrophilic polymer can be compressed to produce tablet forms that, when submerged in aqueous fluids (*i.e.*, gastric fluid), form an outer gel layer [17]. This gel-forming capacity in acidic medium is related to the protonation of surface carboxylic groups. The association of alginate with the anionic CMS is now proposed. This could be a novel and interesting approach to prepare microspheres with low porosity and enhanced gastric stability.

Recently, oral administration forms of diamine oxidase (DAO), formulated as monolithic tablets with CMS and chitosan for colonic delivery, was proposed as an oral antiallergic agent to treat food histaminosis and as a tentative to reinforce the treatment of inflammatory bowel diseases (IBDs) [18, 19].

DAO (EC 1.4.3.22), also known as histaminase, is a copper-containing amine oxidase that exists in plants (pea seedling) [20] and mammals (pig kidney) [21] and possesses a covalently linked cofactor, the topaquinone [22], derived from the post-translational modification of an endogenous tyrosine residue [23]. The enzyme catalyzes the oxidative deamination of the primary amine group of polyamines (putrescine, spermine, and spermidine) and histamine with the release of corresponding aldehydes, hydrogen peroxide, and ammonia. Plant copper-containing amines oxidases have been a point of interest in therapeutic studies because other copper oxidases such as ceruloplasmin [24] and bovine serum amine oxidases have been shown to possess antioxidant [25], cardioprotective [26], and even antitumoral properties [27]. It has also been suggested that pea seedling DAO can reveal an antioxidant-like protection by reducing tissue damage induced by reactive oxygen species generated during the anaphylactic reaction [28]. Furthermore, pea seedling DAO was found to counteract cardiac anaphylaxis and pulmonary asthma-like reaction to aerosolized allergens in sensitized guinea pigs by an antioxidant activity and inactivation of endogenously released histamine [29].

Previous kinetic studies indicated a higher oxidation rate of aliphatic diamines and histamine for vegetal DAO when compared with animal amine oxidase [30]. The efficacy of vegetal DAO to degrade histamine suggests a possible therapeutic application of DAO in the treatment of histamine-related intestinal disorders. Indeed, DAO is the main histamine degrading enzyme in the intestinal tract and is responsible for extracellular histamine degradation by oxidative deamination after mediator release [31]. Food-induced histaminosis can be developed through both an increased presence of exogenous amines (histamine, putrescine, and cadaverine) and a decreased histamine degradation capacity. Symptoms of histaminosis can range from a simple vascular headache [32] to gastrointestinal ailments such as colics, flatulence, and diarrhea. At intestinal tract, high mucosal histamine and a change in mucosal DAO activity have been observed in various dysfunctions including food allergies [33] and IBDs such as Crohn's disease [34] and ulcerative colitis [35]. A lower DAO activity was found on the intestinal mucosa of patients suffering from Crohn's disease or ulcerative colitis [34]. Reduction of inflammation in ulcerative colitis induced in rats was observed after 5 days of intraperitoneal administration of DAO [36], suggesting DAO administration as a useful tool for the treatment of intestinal pathologies.

We are now proposing DAO entrapped in CMS/alginate microspheres for oral administration. The present study is aimed to investigate the capacity of calcium CMS (CaCMS)/alginate complex to form microspheres and to entrap DAO by ionotropic gelation and to evaluate the capacity of microspheres to protect the enzyme in simulated intestinal conditions.

2. Materials and Methods

2.1. Reagents and chemicals

High amylose corn starch was purchased from National Starch (Hylon VII, Bridgewater, NJ, USA). Sodium alginate (Keltone® LVCR, NF grade) was from FMC Biopolymer (Newark, DE, USA). Horseradish peroxidase (Type I, 96 purpurogallin units/mg solid), putrescine (1,4-diaminobutane dihydrochloride), *ortho*-phenylenediaminedihydrochloride, *N*,*N*-diethyl *para*-phenylenediamine, hydrogen peroxide (30%), and pancreatin from porcine pancreas were from Sigma–Aldrich (St-Louis, MO, USA). The chromatographic ion exchange resin YMC-BioPro S75 was from YMC (Japan). Other chemical reagents were ACS grade and used without further purification.

2.2. Preparation of DAO

Lathyrus sativus extract enriched with DAO activity was obtained from 1 kg *L. sativus* seedlings homogenized in 1 L 50 mM phosphate buffer, pH 5.5, containing 400 mM NaCl and squeezed through a liquid filter jute bag. The filtrate was centrifuged at 8,000*g* for 40 Min to remove remaining fibers, and the supernatant was diluted with water to a conductivity of 9 mS (millisiemens). The supernatant was loaded on a 5×8 cm^2 strong exchange column (YMC-BioPro S75) equilibrated with 50 mM phosphate buffer (pH 5.5). After washing with 1 L of the same buffer, the column was eluted with 50 mM phosphate buffer, pH 5.5, containing 300 mM NaCl. The eluate was collected in 50 mL fractions. Those of highest DAO-specific activity were pooled and concentrated to about 10 mL final volume, and then dialyzed in 50 mM phosphate buffer (pH 7.4) overnight. After dialysis, the suspension was centrifuged at 15,000*g* for 15 Min, freezed at −20 °C, and then lyophilized without the addition of a stabilizing agent. The lyophilized samples were stored at −20 °C until used. DAO powder obtained had a specific activity of 2.6 U/mg.

2.3. Preparation of CMS

CMS was synthesized in aqueous medium from high amylose starch as previously described [37]. Briefly, 40 g of starch was dispersed in 100 mL of distilled water under continuous stirring in a jacketed beaker at 55 °C. For gelatinization, a volume of 135 mL of 1.5 M NaOH was incorporated under stirring (1 H) followed by the addition of 30 mL of 10 M NaOH and 15 g of sodium chloroacetate in a minimal volume of water. After 1 H, a volume of 155 mL of distilled water was added to the reaction, which was then neutralized with acetic acid. The CMS was precipitated with 80% methanol and then repeatedly washed with the 80% methanol solution until a conductivity of 50 μS/cm or lower. Finally, it was washed with a methanol/acetone mix (40:60, v/v), air-dried at 40 °C for 24 H, and sieved through a 300 μm screen. The degree of substitution of CMS (DS 0.20) was determined by back titration as described by Assaad et al. [37].

2.4. Preparation of CMS/alginate microspheres

The microsphere-forming solution was prepared by first dissolving CMS (2.5%) in distilled water followed by the addition of sodium alginate to reach 3%. The solution was stirred until the CMS/alginate mixture suspension was uniformly homogenized and without bubble formation. Blank (no enzyme) CMS/alginate microspheres were prepared by ionotropic gelation as described by Patil et al. [38]. A volume of water was added and homogeneously stirred in microsphere-forming solution (1:3, v/v) and transferred into a syringe with a 26G half needle placed approximately 4 cm above 200 mL of chilled calcium chloride ($CaCl_2$) (10%) solution. Microspheres were obtained by dripping the diluted microsphere-forming solution into the $CaCl_2$ solution under stirring. They were left to stabilize in 10% $CaCl_2$. For enzyme immobilization by entrapment in microspheres, a volume of DAO (9 mg/mL) freshly dissolved in 20 mM phosphate buffer (pH 7.2) was added in the CMS/alginate microsphere-forming solution to obtain a final concentration of 3 mg/mL.

Microspheres with entrapped enzyme were prepared as described above by ionotropic gelation. After stabilization, microspheres were withdrawn from the 0.5% $CaCl_2$ solution and placed on a Whatman filter paper inside a vacuum desiccator. Microspheres were then dried under vacuum at 4 °C for 5 days until a constant mass. The collected dry beads presented a loss of approximately 91.7% of their initial wet weight and were stored away from light at 4 °C.

2.5. Determination of entrapment efficiency

Protein encapsulation was verified by 15 Min staining with 0.5% Coomassie brilliant blue G-250 in a methanol/acetic acid/water (40:10:50, v/v/v) solution followed by destaining in a methanol/acetic acid/water (40:10:50, v/v/v) solution. For DAO extraction, 300 mg of microspheres was added to 8 mL of 1 M potassium phosphate buffer (pH 7.2) and incubated at 4 °C until the degradation and precipitation of CMS/alginate microspheres [8]. The supernatant was collected and preserved at 4 °C until enzymatic activity and protein determination assays. Protein concentrations were determined by the Bradford assay using bovine serum albumin as standard, and the protein inclusion yield was expressed as

$$\text{Inclusion yield (\%)} = [\text{Entrapped protein (mg)}/\text{Introduced protein (mg)}] \times 100$$

2.6. Determination of DAO enzymatic activity

The enzymatic activity of DAO entrapped in microspheres was determined using a peroxidase-coupled reaction allowing to quantify the released H_2O_2 as previously described [18]. The assay mixture containing 3.2 mL of 50 mM potassium phosphate buffer (pH 7.2), 50 μL of peroxidase solution (0.1 mg/mL), 250 μL of 30 mM *N*,*N*-diethyl *para*-phenylenediamine solution, and 1 mL of 30 mM putrescine solution was incubated for 5 Min at 37 °C. The assay started with the addition of 300 mg of DAO entrapped in microspheres for the dosage. After incubating for 10 Min at 37 °C, the absorbency was read at 515 nm using an Ocean Optic© fiber optic spectrometer. The standard curve was prepared using various concentrations of H_2O_2 ranging from 0 to 0.4 mM. One enzymatic unit of DAO was considered as the amount of enzyme required to catalyze the oxidation of 1.0 μmol of putrescine per minute at pH 7.2 and 37 °C. The enzymatic activity was also determined for free DAO and DAO after extraction. The previously described assay mixture was incubated for 5 Min at 37 °C and determined by the addition of 500 μL protein sample of DAO (free or extracted from microspheres) for the dosage.

2.7. Evaluation of DAO microspheres in simulated gastric and intestinal fluid

Three hundred milligrams of vacuum-dried beads (containing ~900 μg of proteins) was first left in water for 2 Min for preswelling and washing eventual traces of $CaCl_2$. The DAO

microspheres were then incubated in 10 mL of simulated gastric fluid (SGF, pH 2.0). After 1 and 2 H, samples were withdrawn to detect the retained enzymatic activity of entrapped DAO. Separately, 300 mg of microspheres was incubated in various tubes containing 10 mL simulated intestinal fluid (SIF), pH 6.8, with and without 1% pancreatin [39] under shaking at 50 rpm and 37 °C. At different intervals, supernatants and beads were withdrawn from the tubes and stored at 4 °C until enzymatic activity and protein determination assays.

2.8. DAO zymography on entrapped peroxidase in polyacrylamide gels electrophoresis

Enzymatic detection of DAO by zymography was carried out on peroxidase entrapped in PAGE as described by Calinescu et al. [40]. For the entrapment, 1 mL of horseradish peroxidase (1 mg/mL) was incorporated in the solution of polyacrylamide (final volume of 5 mL), just prior to trigger the polymerization. The stacking gel contained no peroxidase.

2.9. SDS-PAGE

The experiments were conducted using a Mini-Protean® tetra cell (Bio-Rad, Richmond, VA, USA) electrophoresis system. Free DAO and DAO samples extracted from beads (as previously described) were treated (4:1, v/v) with glycerol while the molecular weight standards have been prepared according to the manufacturer's instruction (Bio-Rad). A volume of 30 μL of each sample and 10 μL of molecular weight standards (Broad Range) were loaded and run by SDS-PAGE for 1 H (room temperature, 160 V) on 10% polyacrylamide gel for Coomassie blue staining. For each sample, a comparable volume was run on 10% polyacrylamide gel containing entrapped peroxidase for zymographic revelation of DAO enzymatic activity. The electrophoresis buffer used for both Coomassie staining and zymography runs contained 0.025 M Tris-Base, 0.192 M glycine, and 0.1% SDS.

After electrophoresis, the polyacrylamide (peroxidase-free) gels were incubated for 30 Min in fixation solution containing methanol/acetic acid/water (50:10:40, v/v/v) followed by 1 H staining with 0.5% Coomassie brilliant blue G-250 in a methanol/acetic acid/water (40:10:50, v/v/v) solution. For zymography, the gels containing entrapped peroxidase were rinsed with distilled water and placed in a solution of putrescine (30 mM) for a few minutes followed by the addition of an equal volume of *ortho*-phenylenediamine solution (30 mM) and incubation at 37 ºC for 1 H under agitation in dark.

2.10. Fourier transform infrared spectroscopy

Samples were prepared by mixing vacuum-dried microbeads (50 mg) with dried potassium bromide (150 mg) and compressed in flat-faced punches at 1 T/cm^2 in a Carver (Wabash, IN, USA) hydraulic press and maintained for about 20 Sec. The resulting tablet of 12 mm was then used for Fourier transform infrared (FTIR) analysis [41]. Spectra were recorded (16 scans at a resolution of 2 cm^{-1}) using a Thermo-Nicolet 6700 FTIR spectrometer (Madison, WI, USA) with a deuterated triglycine sulfate-KBr detector and a diamond smart attenuated total reflection platform.

2.11. X-ray diffractometry

One gram of vacuum-dried microbeads was used without modifications or treatment in a Siemens D-5000 diffractometer (Munich, Germany) SOL-X detector and operating in reflectance mode of CoK$_\alpha$ at a wavelength of 1.789 Å, over an angular range of 2θ from 0° to 30° and a scan rate of 2° per minute. The spectra recorded were smoothed using the software Match 2.

2.12. Scanning electron microscopy

One gram of vacuum-dried DAO containing microspheres was treated by incubation in water for 4 H or in SGF with pepsin for 2 H or in SGF with pepsin for 2 H followed by 4 H in SIF containing pancreatin or directly in SIF for 4 and 24 H. Samples were promptly dried using the vacuum-drying procedure. After drying, samples were mounted on metal stubs using double-sided carbon conductive tape and were sputter-coated with gold for scanning electron microscopy (SEM) analysis. Microspheres shape, surface, and diameter were evaluated with a JSM-6010LV InTouchScope™ (SEM) (JEOL, Tokyo, Japan) using a secondary electron image detector. The images were obtained by accelerating voltages of 1.5 kV and high vacuum. The shape and size of microspheres were evaluated on microbeads (n = 6, only one representative for each experimental conditions) and are presented in Fig. 5.

3. Results and Discussion

Alginate was initially selected to immobilize enzyme because of its compatibility, stability, and ease to form microspheres by ionotropic gelation (complexation with bivalent metal ions such as Ca^{2+}) [9]. Microspheres formulated with alginate alone presented a porous structure from which enzyme can promptly diffuse from the microspheres into gastrointestinal media and undesired acidity can diffuse into the microspheres [8]. To prevent these phenomena, the association with other gel-forming polymers such as chitosan, hydroxypropyl cellulose (HPC), or CMS appeared of interest, as it improves the bead structure and reduces its porosity. Chitosan (positively charged) can interact ionically with alginate (with negative charges) to form a polyelectrolyte complex. However, microspheres based on chitosan/alginate presented similar porosity as native alginate and allowed a rapid diffusion of DAO. When combining the HPC (no ionic charges) with alginate, the structure was more compact and the protein was successfully kept inside of the microspheres. However, the mechanical properties of the microspheres were not satisfactory in gastric and intestinal fluids. This is why CMS (negatively charged and hydrogel forming) was used in combination with alginate for co-complexation with calcium. It was supposed that CMS, because of its strong capacity to form a hydrogel and its swelling properties [41], will reduce the porosity of alginate when complexed with calcium. In fact, the mechanical properties of the CaCMS/alginate were definitely improved with a good

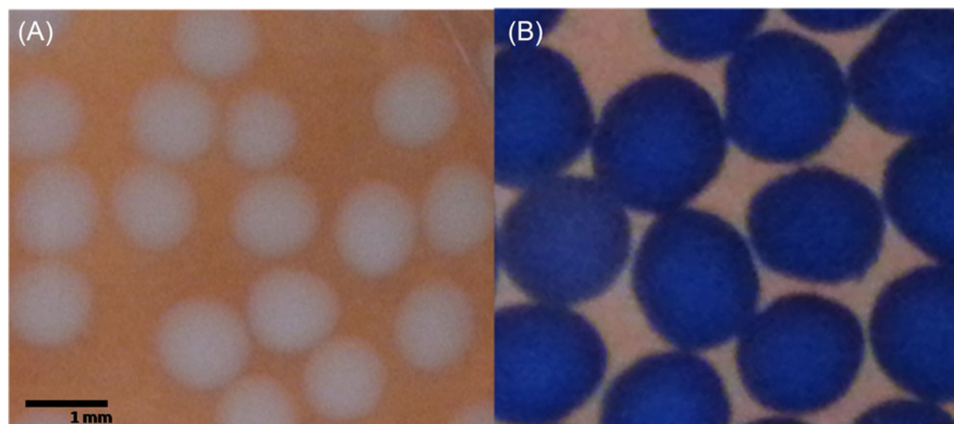

FIG. 1 *Macroscopic image of CMS/alginate microbeads after Coomassie blue staining: (A) without enzyme and (B) with entrapped enzyme.*

stability in biological media and no marked protein diffusion out of microspheres after 24 H. For these reasons, our formulation was based on the complexation of alginate with CMS in the presence of DAO with Ca^{2+}.

3.1. Characterization of microspheres

After optimization, a concentration of 2.5% for CMS and 3% for alginate provided satisfactory mechanical properties and good stability for the microspheres. These results coincide with previous studies that showed that a 2%–3% sodium alginate concentration offered a good immobilization efficiency [13, 42]. At concentrations of CMS greater than 3%, the polymeric solution was difficult to handle because of a high viscosity. Wet microspheres obtained by ionotropic gelation of CaCMS/alginate in the presence of DAO presented predominantly a spherical shape and the enzyme inside microspheres as highlighted by Coomassie blue staining (Fig. 1), whereas enzyme-free microspheres showed no significant coloration. The protein inclusion yield was of about 82%–95% entrapped DAO and the water content was of approximately 91% in wet microspheres. After vacuum drying, the specific enzymatic activity was measured in order to ensure that the chosen entrapment and drying methods did not denaturate the enzyme. The results confirmed the presence of active enzyme in CaCMS/alginate microspheres before and after extraction of protein from microspheres, with a yield of about 45%–60% of the initial activity. The results also suggest a certain porosity affording a permeability of the matrix limited for small molecules only because the substrate, putrescine, is able to rapidly diffuse through the matrix and be oxidized by the enzyme inside the microspheres, whereas the DAO still remains within the beads.

3.2. Electrophoresis studies

In order to further identify the protein profiles, samples of free DAO and of DAO extracted from microspheres were run in SDS-PAGE under nonreducing conditions for protein staining and for zymography. Previous studies indicated the presence of

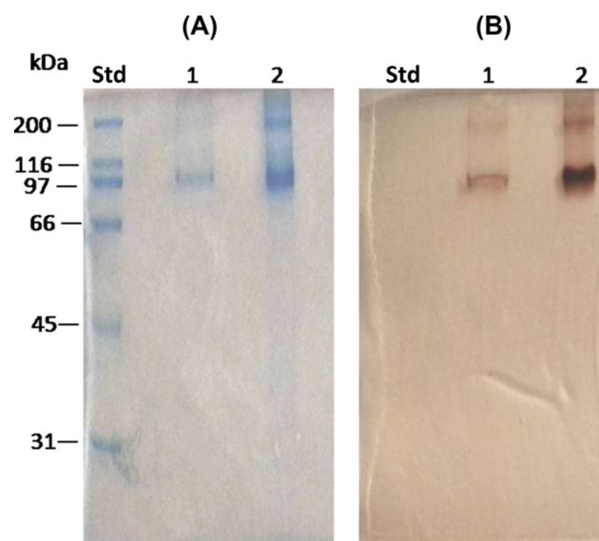

FIG. 2 *SDS-PAGE of diamine oxidase with Coomassie staining (A) and zymography with peroxidase entrapped in 10% polyacrylamide gel (B) of DAO encapsulated in microbeads and extracted with 1 M phosphate buffer (lanes 1) and of free enzyme (lane 2).*

a single band at 72 kDa in denaturating SDS-PAGE [40, 43] for vegetal DAO extracted from *L. sativus* and a band at 95 kDa for DAO extracted from *Pisum sativum* L [44]. The molecular mass of pea seedling amine oxidase was also investigated using the method of gel filtration, indicating a value of 184.0 ± 2.6 kDa [45]. Our Coomassie staining of DAO on nonreducing SDS-PAGE (Fig. 2A) showed bands of similar mobility for both free DAO and DAO extracted from microspheres. Enzyme activity on zymography on peroxidase entrapped in polyacrylamide gel showed bands (Fig. 2B) at the same mobility as those detected by Coomassie staining. The fact that DAO presented activity in zymography confirms that the enzyme remains active after the entrapment and drying process.

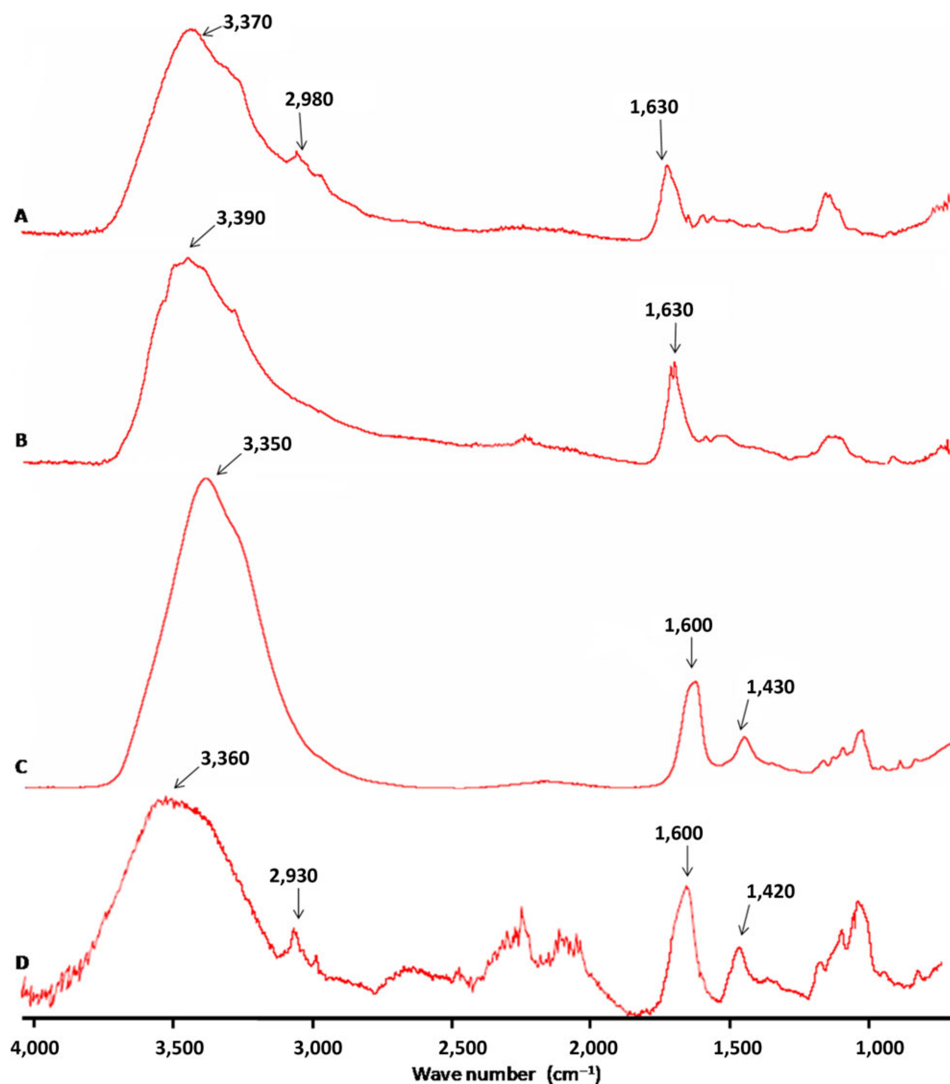

FIG. 3 *FTIR spectra of air-dried microbeads: (A) alginate, (B) enzyme-loaded alginate, (C) CMS/alginate, and (D) enzyme-loaded CMS/alginate.*

3.3. FTIR analysis

FTIR spectra of different types of microspheres loaded and unloaded with DAO are presented in Fig. 3. For calcium alginate beads without DAO, the spectrum showed a broad band assigned mainly for −OH stretching vibration at approximately 3,200–3,400 cm^{-1} and a band at 2,980 cm^{-1} attributed to the alkyl −CH bond of alginate. The band at 1,630 cm^{-1} was related to the asymmetric stretching vibration of the carboxylate group. Similar spectra were observed for calcium alginate microspheres with DAO. However, an increase in the intensity for the bands at 1,630 cm^{-1} may be because of overlapping of stretching vibration from the carboxylate group (alginate) and amide (peptide) bonds of DAO. The combination of CMS and alginate (w/w, 5:6) in microspheres (Fig. 3C) presents a large absorption band located at 3,350 cm^{-1} assigned to the −OH group stretching vibration. This high intensity is probably because of an additional effect of −OH groups from alginate and CMS. The bands at 1,600 and 1,430 cm^{-1} were ascribed to asymmetric and symmetric stretching vibrations of carboxylate groups. In the presence of DAO (Fig. 3D), there is a decrease in the absorption intensity of 3,350 cm^{-1} band (−OH group), probably due to a decrease in the relative amount of –OH groups for dry microspheres. Also, a hydrogen bonding between the DAO and CMS/alginate complex may not be excluded from the larger band at 3,600–2,900 cm^{-1}. The band at 2,930 cm^{-1} (assigned to −CH alkyl) presented an increased absorption, which may be related to the −CH groups of DAO.

3.4. X-ray diffraction analysis

X-ray diffractograms (Fig. 4) of Ca alginate microspheres (Fig. 4A) revealed a major peak at 13° that is associated with the lateral packing of alginate chain [46], another one at 5°–7°, and a shoulder at 8°. The diffractograms for CMS/alginate showed a minor shift of the peak at 13°, which also became more organized and a loss of the peak at 8° (Fig. 4B). Similar but not identical profiles were noticed for DAO entrapped in

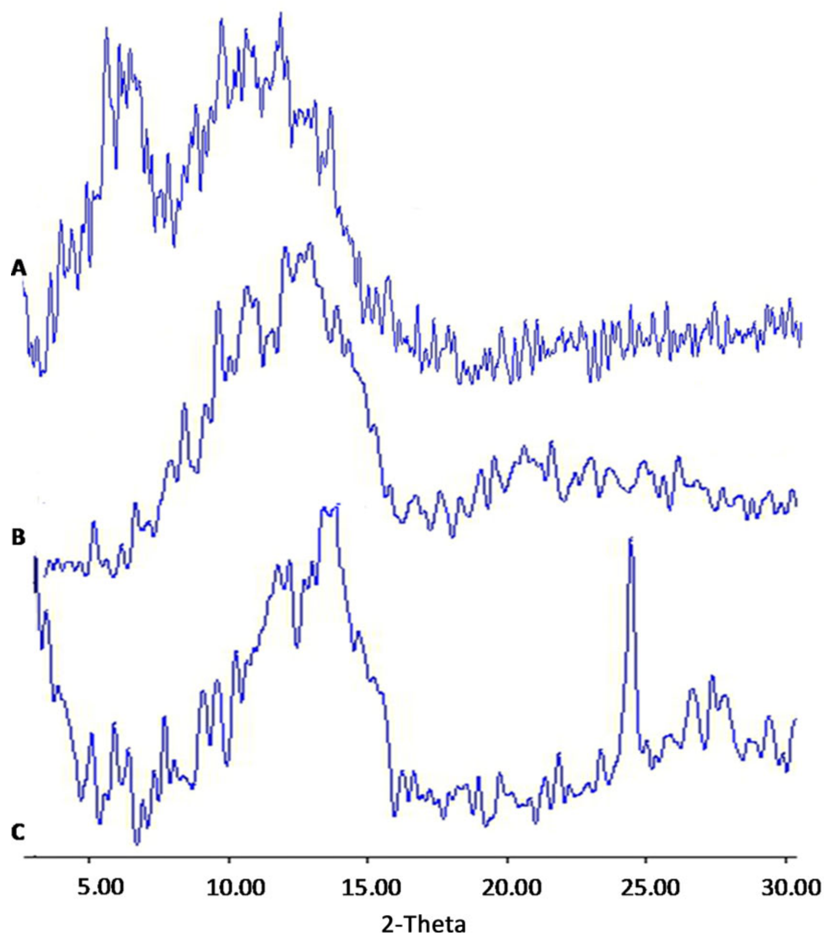

FIG. 4 *X-ray diffraction patterns of (A) alginate microbeads, (B) CMS/alginate microbeads, and (C) CMS/alginate microbeads with entrapped enzyme.*

CaCMS/alginate (Fig. 4C). In addition, the band at 13° seemed more ordered slightly sharper, suggesting a more organized structure, probably because of interactions between carboxylic polysaccharide matrix and DAO enzyme.

3.5. SEM analysis of microspheres

SEM of vacuum-dried CaCMS/alginate microspheres loaded with DAO and following various treatments are presented in Fig. 5. Nontreated microspheres showed a diameter of approximately 1 mm with an almost spherical shape (Fig. 5A). Detailed examination of the surface structure reveals a smooth surface of microspheres, probably because of CMS forming an outer hydrogel. When hydrated (water for 4 H), the microspheres retained the spherical form with a diameter of about 900 μm (Fig. 5C) with a change in the smooth surface for a rough and porous exterior. After 2 H in SGF, a decrease of up to 800 μm in diameter was observed for the spherical microspheres probably because of the protonation phenomenon, which replaces the electrostatic repulsions among carboxylate groups with hydrogen association: carboxyl–carboxyl (dimerization) or carboxyl–hydroxyl [47] and thus a shrinkage is favored [9]. In SGF, the surface appeared more contracted than before the treatment or when treated in water probably because of the tightening of the CMS and alginate gel meshwork [48]. Microspheres incubated in SGF for 2 H and then in SIF for 4 H presented a similar size to that of microspheres incubated only in SGF for 2 H. When treated in SIF with pancreatin for 4 and 24 H, microspheres retained their spherical shape with a size of about 800 μm, but the surface showed a kind of dilatation after 4 H and even more pronounced after 24 H, which may be because of CMS/alginate swelling in neutral or low alkaline pH [49] and to a slow erosion because of, at least in part, alpha-amylase activity of pancreatin.

3.6. Activity of free DAO and of DAO entrapped in microspheres

One of the roles of the enzyme microencapsulation is to maintain its stability in biological fluids, protecting it against gastric acidity and degradation by proteases. Microspheres can be given orally as free-flowing suspensions but the effective dose is not precise enough and thus can lead to failure of treatment. To facilitate oral administration, microsphere dosage forms are often filled within hard gelatin capsules [39]. Furthermore, there are commercially available enteric coatings such as Eudragit® to render capsules resistant against gastric

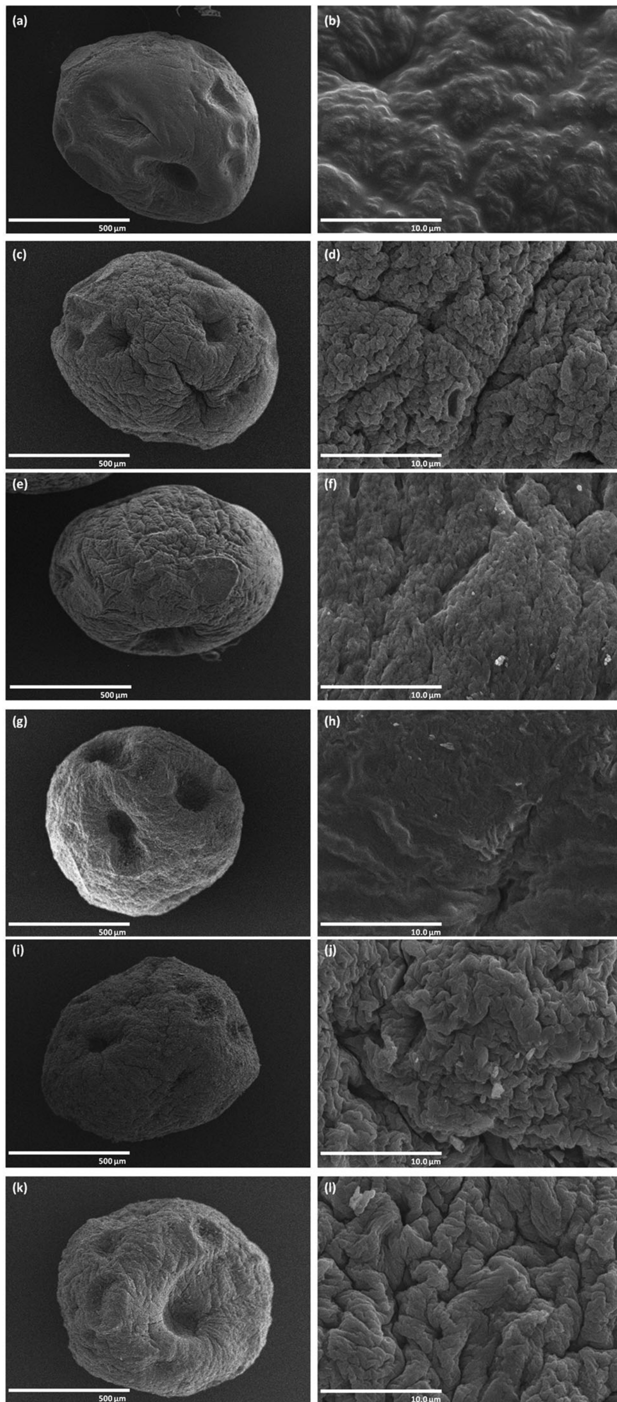

FIG. 5 *Scanning electron micrographs of CMS/alginate air-dried microbeads without any treatment (A), or incubated in water during 4 H (C), in SGF (pH 1.2) for 2 H (E), in SGF for 2 H followed by SIF (pH 7.4) for 4 H (G), in SIF only for 4 H (I), and in SIF for 24 H (K). For each experimental condition, one of microbeads (n = 6) representative for the samples is presented. Surface shapes are shown for each corresponding incubation time (B, D, F, H, J, and L). The enlargements were of 50× for microbeads or of 5,000× for surface shape and were obtained at 10 kV.*

acidity for oral delivery [50]. Our uncoated microspheres have been investigated in SGF and SIF in order to evaluate the physicochemical properties of the CaCMS/alginate complex. Previous studies showed that free DAO is sensitive to gastric acidity [40, 51]. It is also known that alginate microspheres (with porous structure) allow the diffusion of gastric acidity into microspheres inducing a rapid inactivation of enzyme. It was therefore of interest to investigate the stability of DAO entrapped in CaCMS/alginate microspheres against gastric acidity, and a retained activity of approximately 70% was observed after 2 H in SGF at pH 2.0. This conservation of entrapped DAO activity with the CaCMS/alginate microspheres may be because of the high amount of carboxylate groups from both alginate and CMS, which can intercept the access of protons damaging the entrapped enzyme [12]. A similar protection was reported with various peptides and probiotics by Calinescu and co-workers [17, 52] using CMS: monolithic tablets were able to protect sensitive DAO during gastric transit and to release them in the lower intestine tract [18]. Now, it was also of interest to evaluate whether CaCMS/alginate microspheres can protect DAO against proteolytic degradation in SIF.

The retained activities of free and entrapped DAO were tested in SIF at various intervals (Fig. 6). Although DAO is known for possessing a certain resistance to proteolysis in SIF with pancreatin [18], this resistance was limited for a period of about 8 H. The activity of free enzyme in SIF with pancreatin during the first 8 H was of about 80% from the initial activity. Then, its retained activity decreased gradually and was completely lost after 24 H. For DAO entrapped in microspheres, its retained activity was stabilized at about 65% and maintained for whole period of at least 24 H. The decreasing activity of entrapped DAO during the first hour of incubation may be because of several factors such as the loss of the enzyme from the surface of microspheres or a slow hydration and swelling of microspheres, which is necessary for the activation of the enzyme [53, 54] and renders the enzyme accessible to the substrate. These factors explain why the activity of microspheres was initially moderately lower than that of the free enzyme but then remained stable for a longer period of time. These data suggest that the entrapping of DAO in CaCMS/alginate microspheres can afford a protection against proteolysis probably because proteases are unable to diffuse into the microspheres. The role of CMS acting as a filler and increasing the compactness of microspheres appeared to be beneficial for the maintenance of catalytic properties of the entrapped DAO in this novel CMS/alginate matrix.
In conclusion, the entrapment of DAO in CaCMS/alginate microspheres appears as a procedure able to afford protection of the entrapped enzyme against gastrointestinal degradation. Further investigations will be devoted to highlight the possible role of microspheres loaded with DAO orally administered in animal models for the treatment of inflammatory enteric diseases.

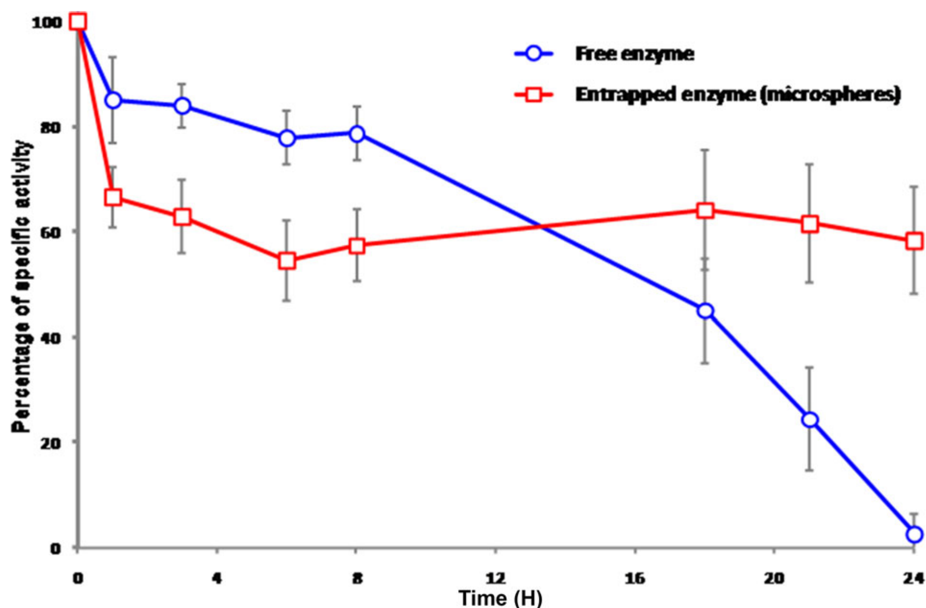

FIG. 6 *Activity of free and entrapped enzyme at various incubation times in SIF (with pancreatin, pH 7.2). The initial specific activity (considered as 100%) of the entrapped enzyme was established as the enzymatic activity of DAO extracted after microbeads treatment in 1 M phosphate buffer (pH 7.2) without pancreatin. For the free enzyme, the 100% was considered the activity determined without modifications and in the absence of pancreatin.*

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5. References

[1] Makadia, H. K., and Siegel, S. J. (2011) Polymers 3, 1377–1397.
[2] Ma, L., and Liu, C. (2010) Colloids Surf. B Biointerfaces 75, 448–453.
[3] Soni, M. L., Kumar, M., and Namdeo, K. (2011) Int. J. Drug Deliv. 2, 64–68.
[4] Tozaki, H., Komoike, J., Tada, C., Maruyama, T., Terabe, A., Suzuki, T., Yamamoto, A., and Muranishi, S. (1997) J. Pharm Sci. 86, 1016–1021.
[5] Haug, A., Larsen, B., and Smidsroed, O. (1966) Acta Chem. Scand. 20, 183–190.
[6] George, M., and Abraham, T. E. (2006) J. Control. Release 114, 1–14.
[7] Tønnesen, H. H., and Karlsen, J. (2002) Drug Dev. Ind. Pharm. 28, 621–630.
[8] Gombotz, W. R., and Wee, S. F. (2012) Adv. Drug Deliv. Rev. 64, 194–205.
[9] Ouwerx, C., Velings, N., Mestdagh, M., and Axelos, M. (1998) Polymer Gels Netw. 6, 393–408.
[10] Draget, K. I., Skjåk-Bræk, G., and Smidsrød, O. (1997) Int. J. Biol. Macromol. 21, 47–55.
[11] Rastogi, R., Sultana, Y., Aqil, M., Ali, A., Kumar, S., Chuttani, K., and Mishra, A. (2007) Int. J. Pharm. 334, 71–77.
[12] Le, T. C., Millette, M., Lacroix, M., and Mateescu, M. A. (2004) Biotechnol. Appl. Biochem. 39, 189–198.
[13] Hung, C.-P., Lo, H.-F., Hsu, W.-H., Chen, S.-C., and Lin, L.-L. (2008) Appl. Biochem. Biotechnol. 150, 157–170.
[14] Anal, A. K., and Stevens, W. F. (2005) Int. J. Pharm. 290, 45–54.
[15] Gotoh, T., Matsushima, K., and Kikuchi, K.-I. (2004) Chemosphere 55, 135–140.
[16] Mulhbacher, J., Ispas-Szabo, P., and Mateescu, M. A. (2004) Int. J. Pharm. 278, 231–238.
[17] Calinescu, C., Mulhbacher, J., Nadeau, E., Fairbrother, J. M., and Mateescu, M. A. (2005) Eur. J. Pharm. Biopharm. 60, 53–60.
[18] Calinescu, C., Mondovi, B., Federico, R., Ispas-Szabo, P., and Mateescu, M. A. (2012) Int. J. Pharm. 428, 48–56.
[19] Mondovi, B., Fogel, W. A., Federico, R., Calinescu, C., Mateescu, M. A., Rosa, A. C., and Masini, E. (2013) Recent Pat. Inflamm. Allergy Drug Discov. 7, 20–34.
[20] McGuirl, M. A., McCahon, C. D., McKeown, K. A., and Dooley, D. M. (1994) Plant Physiol. 106, 1205–1211.
[21] Mondovì, B., Rotilio, G., Costa, M., Finazzi-Agro, A., Chiancone, E., Hansen, R. E., and Beinert, H. (1967) J. Biol. Chem. 242, 1160–1167.
[22] Janes, S., Palcic, M., Scaman, C., Smith, A., Brown, D., Dooley, D., Mure, M., and Klinman, J. (1992) Biochemistry (Mosc) 31, 12147–12154.
[23] Cai, D., and Klinman, J. P. (1994) J. Biol. Chem. 269, 32039–32042.
[24] Atanasiu, R., Dumoulin, M.-J., Chahine, R., Mateescu, M. A., and Nadeau, R. (1995) Can. J. Physiol. Pharmacol. 73, 1253–1261.
[25] Atanasiu, R. L., Stea, D., Mateescu, M. A., Vergely, C., Dalloz, F., Briot, F., Maupoil, V., Nadeau, R., and Rochette, L. (1998) Mol. Cell. Biochem. 189, 127–135.
[26] Mateescu, M. A., and Nadeau, R. (2009) In Copper Amine Oxidase as Antioxydant and Cardioprotectives Agents (Floris, G., and Mondovi, B., eds.). pp. 253–260, CRC Press, Boca Raton , FL.
[27] Averill-Bates, D. A., Agostinelli, E., Przybytkowski, E., Mateescu, M. A., and Mondovi, B. (1993) Arch. Biochem. Biophys. 300, 75–79.
[28] Masini, E., Bani, D., Marzocca, C., Mateescu, M. A., Mannaioni, P. F., Federico, R., and Mondovì, B. (2007) Scientific World J. 7, 888–902.
[29] Masini, E., Cuzzocrea, S., Bani, D., Mazzon, E., Muja, C., Mastroianni, R., Fabrizi, F., Pietrangeli, P., Marcocci, L., and Mondovì, B. (2007) Shock 27, 409–415.
[30] Pietrangeli, P., Federico, R., Mondovì, B., and Morpurgo, L. (2007) J. Inorg. Biochem. 101, 997–1004.
[31] Sessa, A., and Perin, A. (1994) Agents Actions 43, 69–77.
[32] Gallai, V., and Sarchielli, P. (2000) J. Headache Pain 1, 145–154.

[33] Raithel, M., Küfner, M., Ulrich, P., and Hahn, E. (1999) Inflamm. Res. 48, 75–76.
[34] Schmidt, W. U., Sattler, J., Hesterberg, R., Röher, H. D., Zoedler, T., Sitter, H., and Lorenz, W. (1990) Agents Actions 30, 267–270.
[35] Mennigen, R., Kusche, J., Streffer, C., and Krakamp, B. (1990) Agents Actions 30, 264–266.
[36] Fogel, W. A., and Lewinski, A. (2006) Inflamm. Res. 55, S63–S64.
[37] Assaad, E., Blemur, L., Lessard, M., and Mateescu, M. A. (2012) J. Biomater. Sci. Polym. Ed. 23, 1713–1728.
[38] Patil, P., Chavanke, D., and Wagh, M. (2012) Int. J. Pharm. Pharm. Sci. 4, 27–32.
[39] Convention, U. S. P. (2009) USP 32 NF 27: United States Pharmacopeia National Formulary. Supplement 2, United States Pharmacopeial Convention Inc., Rockville.
[40] Calinescu, C., Federico, R., Mondovi, B., and Mateescu, M. A. (2010) Anal. Bioanal. Chem. 396, 1281–1290.
[41] Assaad, E., Wang, Y. J., Zhu, X. X., and Mateescu, M. A. (2011) Carbohydr. Polym. 84, 1399–1407.
[42] Quiroga, E., Illanes, C. O., Ochoa, N. A., and Barberis, S. (2011) Process Biochem. 46, 1029–1034.
[43] Federico, R., Cona, A., Caliceti, P., and Veronese, F. M. (2006) J Control Release115, 168–174.
[44] Ebrahimnejad, H., Gheisari, H., and Khan Nazer, A. (2013) J Food Process. Technol. 4, 2.
[45] Kivirand, K., and Rinken, T. (2007) Proc. Estonian Acad. Sci. Chem. 56, 164–171.
[46] Vreeker, R., Li, L., Fang, Y., Appelqvist, I., and Mendes, E. (2008) Food Biophys. 3, 361–369.
[47] Assaad, E., and Mateescu, M. A. (2010) Int. J. Pharm. 394, 75–84.
[48] Kim, C.-K., and Lee, E.-J. (1992) Int. J. Pharm. 79, 11–19.
[49] Pasparakis, G., and Bouropoulos, N. (2006) Int. J. Pharm. 323, 34–42.
[50] Sonaje, K., Chen, Y.-J., Chen, H.-L., Wey, S.-P., Juang, J.-H., Nguyen, H.-N., Hsu, C.-W., Lin, K.-J., and Sung, H.-W. (2010) Biomaterials 31, 3384–3394.
[51] Federico, R., Befani, O., Mondovi, B., Mulhbacher, J., and Mateescu, M. A. (2000) Inflamm. Res. 49, S60–S61.
[52] Calinescu, C., and Mateescu, M. A. (2008) Eur. J. Pharm. Biopharm. 70, 582–589.
[53] Wilmot, C. M., Murray, J. M., Alton, G., Parsons, M. R., Convery, M. A., Blakeley, V., Corner, A. S., Palcic, M. M., Knowles, P. F., and McPherson, M. J. (1997) Biochemistry (Mosc) 36, 1608–1620.
[54] Su, Q., and Klinman, J. P. (1998) Biochemistry (Mosc) 37, 12513–12525.