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RESEARCH PAPER

Novel Spray-Dried Genipin-Crosslinked Casein Nanoparticles for Prolonged Release of Alfuzosin Hydrochloride

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ABSTRACT

Purpose To propose a simple method for the development of genipin-crosslinked casein micelles as a new delivery platform for prolonged release of alfuzosin hydrochloride.

Methods Crosslinked casein micelles entrapping alfuzosin were transformed into solid redispersible nanoparticles *via* spray-drying technique with no need for drying adjuvants based on the stabilizing effect of casein.

Results The nanoparticles displayed high production yields (86.99–94.63% *w/w*) with a reasonable drug incorporation efficiency ranged from 92.86 to 97.75%. The nanoparticles were readily reconstituted in aqueous solution with a particle size range of 122.1–260.0 nm and a zeta potential range of −21.6 to −36.6 mV indicating a good colloidal stability. No drug crystals were detectable in the scanning electron micrographs revealing successful encapsulation of alfuzosin into casein nanoparticles which was confirmed by differential scanning calorimetry. The nanoparticles succeeded in prolonging the drug release that could be controlled by modulating the genipin crosslinking degree. The release data showed a good fit into Higuchi release kinetics with non-Fickian type of drug diffusion.

Conclusions These results demonstrated that genipin-crosslinking combined with spray-drying technique could be used as a promising tool to develop solid redispersible casein nanoparticles with sustained drug release properties.

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INTRODUCTION

Benign prostatic hypertrophy (BPH) is a condition characterized by a nodular enlargement of prostatic tissue leading to obstruction of the urethra. American health care policy and research (AHCPR) guidance recommended α-blockers as a first-line therapy for BPH (1). Alfuzosin (ALF), a quinazoline derivative, is a selective and competitive α_1-adrenoceptor antagonist approved by FDA for the treatment of symptomatic prostatic hyperplasia (2). It distributes preferentially in the prostate, compared with plasma, and decreases the sympathetically controlled tone of prostatic smooth muscle causing relaxation of smooth muscles and improvement in urine flow. ALF HCl is a highly water soluble drug that is readily absorbed after administration with a short half-life (2,3).

Application of nanotechnology in drug delivery systems has opened up new areas of research in sustained release of various drugs (4). Sustained release of the drug from the nanoparticles maintains the therapeutic concentration for long durations. However, the industrial development of nanoparticulate suspensions is limited due to the problems in maintaining stability of suspensions for a prolonged time period (5). In order to improve stability, studies have been dedicated to develop solid forms of polymeric colloidal systems. Freeze-drying technique was successfully used to dry polymeric nanoparticulate systems with the conservation of original properties after rehydration. However, this technique is highly expensive and thus reserved for products with a high added value (6). Then, spray-drying process appeared as an interesting alternative to freeze-drying in order to develop dry powdered nanoparticles (7).

Spray-drying technique is extensively used in the pharmaceutical field since it allows the preparation of dry powders with specific characteristics such as particle size and shape (8). In addition, formulation processes including encapsulation, complex formation and even polymerization can be accomplished in a single step (9). This method offers advantages such as rapid processing, applicability to heat-sensitive materials and the possibility of scale-up with a low amount of residual solvent in the final product (8,9). In addition, spray-dried powders that exhibit sustained drug release properties may be generated through the inclusion of drug release modifiers such as hydroxypropyl cellulose, glyceryl behenate and polylactic acid (10). Spray-drying technique has been employed to dry nanoparticles improving their physico-chemical stability (7–9). Amorim *et al.* (11), developed nanoparticles based on chitosan and N-carboxymethylchitosan crosslinked with tripolyphosphate (TPP) by co-spray drying with the antioxidant drug idebenone. A sustained release pattern of the drug from the nanoparticles was observed due to the inner location of idebenone with diffusion toward the medium.

Casein (CAS), the major milk protein, is inexpensive, readily available, non-toxic and highly stable. As a natural food product, this GRAS (generally recognized as safe) protein is biocompatible and biodegradable (12). Many of the structural and physicochemical properties of CAS facilitate its functionality in drug delivery systems (13). These properties include binding of ions and small molecules, exceptional surface-active and stabilizing properties, excellent emulsification and self-assembly properties together with superb gelation and water binding capacities. The pH-responsive gel swelling behavior renders CAS useful for programmable drug release (12,13). CAS has been widely studied for sustained delivery of cytotoxic drugs from microparticle formulations crosslinked with glutaraldehyde (14). Theophylline-loaded CAS microspheres crosslinked with glutaraldehyde were found to be resistant to proteolytic tract and were found to sustain theophylline release for more than 24 h following single oral administration in rabbits (15). Furthermore, CAS was considered a good candidate for controlled release parenteral formulations. It was demonstrated that 50 to 60% of the incorporated progesterone was released in phosphate buffer from glutaraldehyde-crosslinked CAS microspheres in about 30 days and then after attained a steady state release (16). These studies suggests CAS to be a promising carrier for the sustained release of many orally as well as parenterally administered drugs.

In recent years, CAS-based nanovehicles were utilized for drug and nutraceutical delivery applications. CAS micelles were successfully used as a protective vehicle for vitamin D_2 (17) and docosahexaenoic acid (18) against UV-light-induced degradation and oxidation, respectively. They also enhanced the solubility and cytotoxicity of curcumin to human leukemia cell line (19). Shapira *et al.* (20), showed that β-CAS nanomicelles could entrap and deliver hydrophobic chemotherapeutics such as mitoxantrone and paclitaxel allowing them to be thermodynamically stable In aqueous solutions for oral-delivery applications in gastric carcinoma. Recently, Bachar *et al.* (21), successfully developed celecoxib-loaded β-CAS nanomicelles with high encapsulation loads for oral delivery.

Although the use of glutaraldehyde as a crosslinker leads to improvement of the mechanical properties and stability of CAS, its high toxicity may limit the applications of the final product. Therefore, the use of non-toxic crosslinking agents was emerged as an alternative (22). Genipin is a naturally occurring crosslinking agent extracted from the gardenia fruit with a negligible cytotoxicity (~10,000 times less than glutaraldehyde) (23). A novel CAS-based hydrogel for the controlled release of bovine serum albumin has been prepared using genipin to crosslink CAS protein in an aqueous system (24). The mechanical strength of the crosslinked CAS hydrogel could be tuned by the amount of genipin.

In this study, we demonstrate for the first time that spray-dried formulations of CAS as a nanoparticle matrix and genipin as a crosslinker generate highly dispersible nanoparticulate powder that exhibit sustained release properties of alfuzosin hydrochloride without using any drying adjuvants based on the stabilizing action of CAS itself.

MATERIALS AND METHODS

Materials

Alfuzosin hydrochloride (ALF) was obtained as a gift sample from Amriya Pharmaceutical Industries Co., PHARCO Corporation (Alexandria, Egypt). Casein (CAS) from bovine milk was purchased from Sigma-Aldrich (St. Louis, USA). Genipin >98% was purchased from Synsci Pharmaceutical Co., Ltd. (China). Sodium azide was from LOBA Chemie Pvt., Ltd. (Mumbai, India). All other chemicals were of analytical grade and used without further purification.

Preparation of ALF-Loaded CAS Nanoparticles

Casein (CAS) solution was prepared by dissolving CAS in 0.1 N sodium hydroxide solution then its pH was adjusted to 7.4 with 1 N HCl. A calculated amount of ALF was dissolved in the CAS solution under moderate magnetic stirring for 2 h. The solid nanoparticles were then obtained by spray-drying of this solution using a Büchi B-290 Mini-Spray Dryer (Flawil, Switzerland), equipped with a high-performance cyclone, a two-component nozzle and current flow, with inlet temperature of 150°C, outlet temperature of 90°C, aspiration air of 90%, feed flow of 5 mL/min,

spraying pressure of 5.0–5.8 mbar and air flow rate of 320 L/h. Unloaded CAS nanoparticles (F_0) were prepared the same as above but without using drug. For the preparation of genipin-crosslinked ALF-loaded CAS nanoparticles; genipin solution in ethanol, in the required concentration, was added portionwise to the ALF-CAS solution under magnetic stirring for the specified crosslinking time. The spray-dried nanoparticles were then stored in a desiccator at 25°C until further analysis.

Characterization of Spray-Dried CAS Nanoparticles

Process Yield and Moisture Content

The residual moisture content of the spray-dried powders was measured by coulometric Karl Fischer titration (756 KF Coulometer equipped with a 774 Oven sample processor, Metrohm, Switzerland) at an oven temperature of 150°C. Dehydrated methanol (20 mL) was titrated to the electrometric end point with the KF reagent. The nanoparticle sample was then carefully transferred to the titration vessel and after stirring for 1 min titrated again using the KF reagent till the characteristic end-point. The weights of the spray-dried powders collected were corrected according to their moisture content. The yield was calculated by dividing these quantities by the total mass introduced in the preparation submitted to drying.

Drug Content in the Nanoparticles

An aliquot of accurately weighed 50 mg of each batch of the spray-dried nanoparticles was completely dissolved in 50 mL of 0.1 N sodium hydroxide solution. This solution was filtered through a 0.22 μm membrane filter and the amount of ALF dissolved in the medium was determined by UV spectrophotometery (T80, UV/VIS Spectrometer, PG Instruments Ltd., UK) at 244 nm. An equal mass of unloaded CAS nanoparticles was treated in the same manner as drug-loaded nanoparticles to be used as a blank solution. The percentage loading capacity (%LC) and incorporation efficiency (%IE) for each formula were calculated using the following equations:

$$\%\ \mathrm{LC} = (\text{Mass of drug in nanoparticles} / \text{Mass of nanoparticles recovered}) \times 100. \quad (1)$$

$$\%\ \mathrm{IE} = (\text{Mass of drug in nanoparticles} / \text{Mass of drug used in formulation}) \times 100. \quad (2)$$

Particle Size and ζ-potential

Particle size of the reconstituted freshly prepared spray-dried nanoparticles, as well as after 6 months srorage, was measured using a dynamic light scattering (DLS) technique with a NanoZS/ZEN3600 Zetasizer (Malvern, Instruments Ltd., Malvern, UK). This system is equipped with a 4 mW helium/neon laser at 633 nm wavelength and measures the particle size with the noninvasive backscattering technology at a detection angle of 173° after an at least 200-fold dilution with purified water. All of the DLS measurements were performed at 25.0±0.1°C at 20 s intervals for three repeat measurements. The Z-average was calculated from the autocorrelation function of the intensity of light scattered from the particles. For the zeta potential measurement, each diluted nanoparticle suspension (1 mL) was put in a universal folded capillary cell equipped with platinum electrodes. The electrophoresis mobility was measured and the zeta potential ζ was calculated by the Dispersion Technology Software provided by Malvern.

Morphological Analysis

Morphological evaluation of spray-dried nanoparticles was carried out using a model JFC-1100E scanning electron microscope (JEOL, Japan). The diluted nanoparticle suspensions were poured into a cover glass to evaporate the water medium. After evaporation, standard coating by Au-Pd spattering in a SPI-MODULE sputter coater for 2 min under vacuum made the specimen electrically conductive. Scans were performed at an acceleration voltage of 20 kV.

Differential Scanning Calorimetry (DSC)

To investigate the physical state of ALF inside CAS nanoparticles, thermograms of CAS, ALF, selected unloaded and ALF-loaded CAS nanoparticles were recorded by DSC 6 differential scanning calorimeter (Perkin Elmer, USA). Samples (2–4 mg) were placed in sealed aluminum pans and heated at 10°C/min under a nitrogen atmosphere (flow rate 20 mL/min) in the range of 30–300°C. An empty aluminum pan was used as a reference.

Fourier Transform Infrared (FTIR) Spectroscopy

The FTIR spectra of CAS, ALF and ALF-loaded CAS nanoparticles were recorded using a Spectrum RXI FT-IR spectrometer (Perkin Elmer, USA). Samples were finely ground with infra-red grade KBr then pressed into pellet

and IR spectra were taken in transmission over the range of 4000–500 cm^{-1} at ambient temperature.

In Vitro Release of ALF from CAS Nanoparticles

In vitro release of ALF from the drug-loaded CAS nanoparticles and drug solution was assessed using the dialysis bag method. Pure drug and drug-loaded nanoparticles (eq. to 10 mg ALF) were dispersed in phosphate-buffered saline (PBS, pH 7.4) and placed in a cellulose ester dialysis membrane (Cut-off 12–14 kDa) sealed with appropriate universal closures (Spectrum Laboratories, Inc., California, USA). The bags were then tied to the paddle of USP XXIV dissolution apparatus II (Type PTWS3, Pharma Test, Germany) and dialyzed against 900 mL PBS (pH 7.4) containing 0.02% sodium azide as a preservative. The entire system was incubated at 37±0.5°C under stirring at 100 rpm. At designated time intervals, 5 mL of the release medium was removed and replaced with the same volume of fresh PBS solution. All samples were filtered through a 0.45 µm membrane filter and the amount of ALF released was analyzed by UV spectrophotometery at 244 nm. For evaluation of release kinetics, the obtained release data were fitted into first order, zero order and Higuchi equations. Selection of the best model was based on the comparisons of the relevant correlation coefficients. To understand the release mechanism, the release data were subjected to the kinetic analysis using Korsmeyer–Peppas model correlating drug release to time by the simple exponential equation for the fraction of drug release < 0.6 (25):

$$M_t/M_\infty = kt^n \quad (3)$$

Where (M_t/M_∞) is the proportion of drug released at time t, (k) is the kinetic constant and the release exponent (n) has been proposed as indicative of the drug release mechanism.

Statistics

All measurements were carried out in triplicate and values are presented as the mean±S.D. Statistical significance is analyzed using Student's *t*-test. Differences between experimental data are considered significant when P-value is less than 0.05.

RESULTS AND DISCUSSION

Spray-Drying Process and the Stabilizing Effect of Casein

Spray-drying is a friendly technology for transforming liquid feed into a dried particulate form by a one-step process (26). Compared to freeze-drying, spray-drying takes less time and is a cheaper process (6,7). Nevertheless, spray-drying requires particular attention in the process control because of high number of parameters and limitations such as efficient particle collection and the potential instability of highly sensitive materials (9,26). In order to maintain the advantages of colloidal carriers, the nanoparticulate dispersions should be reconstituted with their original properties. Several dispersed systems such as emulsions (27), liposomes (28), nanospheres (8,11) and nanocapsules (7–9) were successfully spray-dried with the preservation of their structure using drying-aid agents *e.g.* lactose, hydroxypropylmethylcellulose and silicon dioxide (Aerosil 200®) (9). Without drying adjuvant, it was not possible to spray-dry the suspensions due to the strong adhesion of the product on the spray-dryer walls (29). The drying-aid molecules are expected to protect the nanoparticles, not only from the heat but also against particle/particle interactions able to involve their aggregation. Thus, stabilizing adjuvants added to the spray solution are always necessary to improve process- and storage-stability (9,29).

As milk proteins are very perishable products, they are often converted by spray-drying into stable products such as powders (30). Caseins are proline-rich, open-structured rheomorphic proteins. The proline peptides in the CAS structure tend to interrupt alpha-helix and beta strands. As a result, CAS has a relatively little secondary or tertiary structure, therefore caseins are heat stable (12,13). In the present study, the spray-drying technique was used for the nanoparticle suspension conversion into redispersible solid nanoparticles. No drying adjuvant was needed for efficient spray-drying depending on the stabilizing properties and thermal stability of CAS itself. A minimal powder adhesion to spray-dryer walls was observed thus enabled the collection of high yield powders. Moreover, the spray-dried nanoparticles were easily reconstituted in aqueous solution exhibiting a particle size in the nano-range with no coagula observed in solution after reconstitution. These results suggest the protein itself may act as a drying auxiliary. The stabilizing effect of CAS as a spray-drying additive has been previously investigated by Wang *et al.* (31).

The use of high performance cyclone enabled the collection of high yields of the spray-dried powders (86.99–94.63% *w/w*) (Table I). Karl-Fisher analysis of the spray-dried powders indicated that the moisture content of the powders ranged from 2.1 to 4.9% *w/w*. This water retained in the CAS matrix during spray-drying may be due to the water binding capacity of CAS. Even so, these values are in line with other studies that reported the moisture content of spray-dried powders to be up to 7.5% *w/w* (10).

Drug Loading to CAS Micelles

Analysis of the ALF content of the spray-dried nanoparticles indicated that the drug incorporation efficiency ranged from

Table I Composition and Physicochemical Properties of Spray-Dried CAS Nanoparticles (values are the mean±SD, $n = 3$)

Formula	Variable	Process yield (%w/w)	Drug content (%w/w)	Incorpor. efficiency (%w/w)	Particle size (nm)	Zeta potential (mV)
	ALF/CAS mass ratio[a]					
F_0	-	94.63	-	-	110.7±3.36	−36.1±2.46
F_1	1:3	93.82	20.86±1.35	97.75±2.58	260.0±4.22	−21.6±1.57
F_2	1:5	89.04	10.99±0.59	94.71±4.49	189.7±6.29	−35.4±3.53
F_3	1:7	90.25	9.34±1.32	95.64±1.35	122.1±2.07	−36.6±4.03
	Genipin conc.[b](% w/w)					
F_4	2.5	94.15	20.17±0.51	94.97±2.44	208.3±5.60	−34.8±1.23
F_5	10	92.58	19.37±1.14	93.35±3.37	206.2±3.23	−34.2±2.40
F_6	40	86.99	16.01±0.47	92.86±3.18	198.7±3.45	−36.4±3.02
	Genipin crosslinking time [c] (hr)					
F7	1	87.33	19.45±0.25	92.93±5.28	220.5±4.47	−36.2±3.78
F_5	3	92.58	19.37±1.14	93.35±3.37	206.2±3.23	−34.2±2.40
F_8	5	88.52	18.46±0.04	95.22±4.29	181.5±4.63	−35.7±2.23

F_0-F_3 nanoparticles that are not crosslinked with genipin

[b] F_4, F_5 and F_6 were prepared using 1:5 ALF/CAS mass ratio and 3 h genipin-crosslinking time

[c] F_7, F_5 and F_8 were prepared using 1:5 ALF/CAS mass ratio and 10% w/w genipin

92.86 to 97.75% and the drug loading capacity from 9.34 to 20.86% (Table I). Amorim *et al.* (11), showed a lower incorporation efficiency of idebenone (56.5–99.1% and 64.9–84.8%) in chitosan and N-carboxymethylchitosan spray-dried nanoparticles crosslinked with TPP, respectively. The authors indicated that the drug was not completely incorporated into the polymer particles during drying, probably because of sticking of idebenone on the wall of the spray dryer due to its low melting point (53°C) (11).

Caseins are amphiphilic proteins that can be thought as block copolymers with high levels of hydrophobic and hydrophilic amino acid residues. Therefore, caseins exhibit a strong tendency to self-assemble into spherical CAS micelles (12,13). ALF is a low-molecular-weight and mildly lipophilic drug ($\log D_{octanol/water}$ =1.5 at pH 7.40) which can partition in cell membranes. However, ALF is a weak base (pK_a=8.13), present mainly in ionized form (approx. 80%) at pH 7.4 (32). In our study, ALF was loaded into the CAS micelles then crosslinked with genipin before they were transformed into nano-powdered form by spray-drying technique. By adjusting the pH of CAS solution at 7.4 *i.e.* above its isoelectric point pI (4.6–4.8), the carboxylic and phosphate groups of CAS become negatively charged and so can attract the positively charged amino groups of ALF. Thus, we owe the successful drug binding to CAS to both hydrophobic interactions within the hydrophobic core of CAS micelles besides the electrostatic attractions between the negatively charged CAS and the positively charged drug molecules.

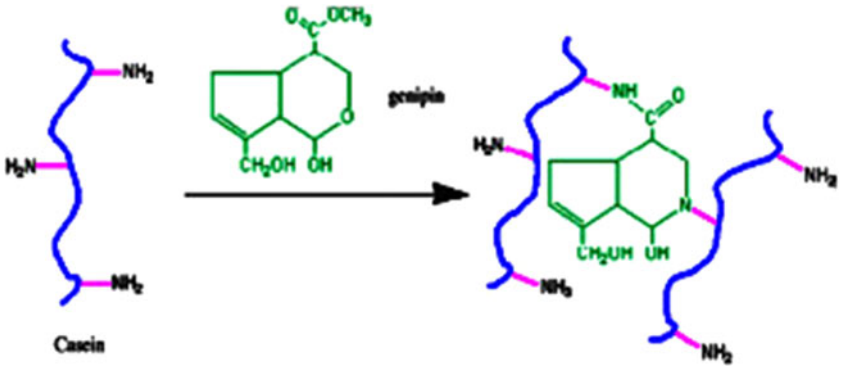
Crosslinking Mechanism

Chemical crosslinkers (*e.g.* glutaraldehyde and formaldehyde) are usually used to harden protein nanoparticles to achieve the desired sustained drug release properties. However, these compounds suffer from the presence of residual unreacted crosslinker inside the nanoparticles together with the risk of formation of toxic products by reaction with the tissues during *in vivo* biodegradation (22,23). Therefore, the use of non-toxic crosslinking agents (*e.g.* genipin and transglutaminase) was emerged as an alternative to glutaraldehyde. Genipin reacts with primary amine groups in the presence of oxygen thus it is an effective crosslinking agent for proteins containing amino groups and has been found to be much less cytotoxic than glutaraldehyde (33).

In this work, genipin was used to crosslink drug-loaded CAS micelles before spray drying. The crosslinking reaction was proposed by Song *et al.* (24), to involve two free amino groups of lysine residue on the CAS macromolecular chains crosslinking with one molecule of genipin (Scheme 1).

To confirm this crosslinking reaction, photo images were taken for the ALF-loaded CAS micellar solution crosslinked with genipin before spray-drying for different crosslinking times (Fig. 1). It was found that the color darkened with the increasing of genipin crosslinking time. After adding genipin, the color of the CAS solutions turned gradually from transparent to yellow, brown and finally to dark blue. This blue coloration is attributed to double bonds of the genipin-crosslinking molecules (24,33).

Scheme I A possible crosslinking mechanism for the reaction of casein with genipin in aqueous system (26).



Characteristics of Spray-Dried Nanoparticles

Dynamic light scattering measurements showed that the spray-dried ALF-loaded CAS nanoparticles exhibited a particle size in the range of 122.1–260.0 nm (Table I) with a relatively large polydispersity (0.34–0.45) which could be correlated to the size and composition of CAS micelles (50–500 nm in diameter, 150 nm average diameter). These findings were almost the same after 6 months storage. Caseins as natural proteins are mixtures of mainly four phosphoproteins, αS_1-, αS_2-, β-, and κ-CAS with a range of molecular weights between 19 and 25 kDa thus producing heterogeneous nanoparticle size distribution (12,13). Figure 2a. revealed a bimodal size distribution of ALF-loaded CAS nanoparticles (F_3). The first peak was obtained at 135.2 nm diameter, apparently reflecting CAS micelles and representing 91.9% of the particles. The second peak was at 9.7 nm diameter, apparently monomeric protein molecules, and it represented 8.1% of the particles. Similar bimodal distributions were obtained at different ALF/CAS formulations. Shapira *et al.* (34), showed that paclitaxel-β-CAS and vinblastine-β-CAS micelles had approximately mean diameters of around 100 nm and 150 nm, respectively. At a higher drug loading, particle sizes rose to approximately mean diameters of 200 nm and 260 nm, respectively, and the particle size distributions were either mono- or bi-modal for both drugs. In the study conducted by Gaiani *et al.* (30), the hydrodynamic diameter of the rehydrated spray-dried CAS powders was found to be around 210 nm, that may correspond to CAS micelle size and the suspension was too polydisperse.

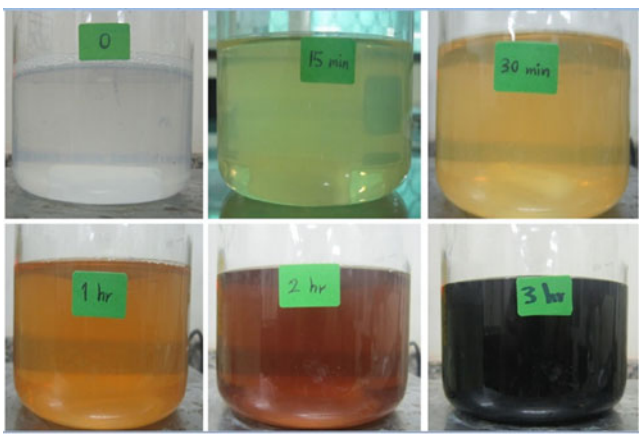


Fig. 1 The color change of genipin-crosslinked ALF-loaded CAS micellar solution with time before spray-drying.

From Table I, it is also clear that decreasing the drug/protein ratio from 1:3 to 1:7 resulted in a corresponding decrease in particle size from 260.0 to 122.1 nm, respectively. As expected, the size of nanoparticles could be decreased with increasing either genipin concentration or crosslinking time. This can be correlated with a higher extent of genipin crosslinking density showing the possibility to modify particle size by this method.

Zeta potential measurements of spray-dried ALF-loaded CAS nanoparticles are presented in Table I. The nanoparticles were negatively charged with a zeta potential range of −34.2 to −36.6 mV indicating a good colloidal stability except those with ALF/CAS ratio of 1:3 that showed a value of −21.6 mV (Fig.2b). This negative charge is a result of the net electrostatic charge on CAS surface at pH 7.4, which is above its isoelectric point, where the CAS carboxylic groups become negatively charged. At the ALF/CAS ratio of 1:7 and 1:5, the zeta potential remained rather constant around −35 mV equal to that of pure negatively charged unloaded CAS nanoparticles (F_0) suggesting that ALF entrapment within the particles core is favorable. However, as the drug/protein ratio increased to 1:3, the zeta potential increased to a value of −21.6 mV due to the positive charge on ALF at pH 7.4. Apparently ALF first binds to the core of CAS micelles mainly by hydrophobic interactions and when the hydrophobic core is fully loaded, it starts electrostatically binding to the negatively charged CAS particle surface, whose charge is dominated by the serine-phosphate groups in the hydrophilic N-terminal domain resulting in an increase in zeta potential. Similar results were reported for mitoxantrone-β-CAS micelles that showed zeta potentials below −40 mV. However, as the drug/CAS ratio was raised, the zeta potential started rising because of the positive charge of mitoxantrone at pH 7 (34). From Table I, it

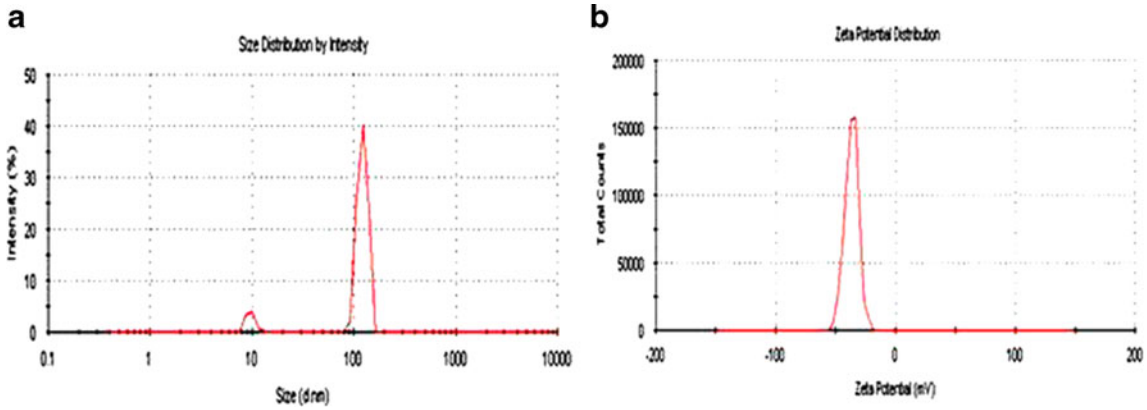


Fig. 2 Particle size (**a**) and zeta potential (**b**) distributions of ALF-loaded CAS nanoparticles (F_3).

can be seen that genipin-crosslinking did not affect the surface charge of the nanoparticles.

Morphology of the reconstituted spray-dried drug-loaded CAS nanoparticles (F_3) was revealed by scanning electron microscopy (Fig. 3). The nanoparticles were spherical and homogeneous with a diameter around 120 nm which corroborates with DLS measurements. No drug crystals were detected revealing that ALF was encapsulated in the nanoparticles in an amorphous nature; this was as expected, as powders generated through spray-drying are known to be predominately amorphous (35).

Solid-State Properties

The physical status of ALF formulated in the spray-dried CAS nanoparticles was compared with the free drug (Fig. 4). ALF in its natural state exists as crystals, which are characterized by the high melting peak around 235°C (Fig. 4b). CAS thermogram displayed a broad endothermic peak at 94.8°C due to the evolution of water from the sample and another one around 200°C (Fig. 4a). However, when the drug was formulated in CAS nanoparticles, the peak at its original melting point disappeared, and the thermogram was essentially similar to that of unloaded CAS nanoparticles (Fig. 4c and d) suggesting that ALF encapsulated in spray-dried CAS nanoparticles existed in the amorphous or in the solid solution state thus confirming the results of morphological analysis. Puthli and Vavia (36), showed that CAS microparticles loaded with levonorgestrel exhibited a thermal profile similar to that of the unloaded ones with no peak of drug melting being detected in the loaded CAS microparticles. The authors explained this by the total incorporation of the drug into the microparticles suggesting a molecular dispersion of drug inside the system. For cimetidine-loaded chitosan microspheres prepared by spray drying, the drug peak disappeared indicating that the drug was molecularly dispersed inside the matrix of chitosan as a solid solution (37). These results, taken together, suggest that the nano-encapsulation and spray-drying process produce a marked decrease in crystallinity of ALF and/or confers to this drug a nearly amorphous state.

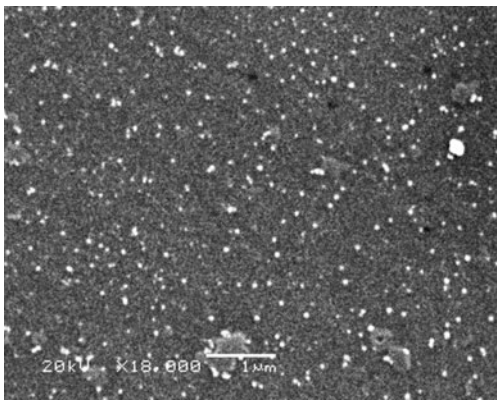


Fig. 3 Scanning electron micrograph of reconstituted spray-dried ALF-loaded CAS nanoparticles (F_3).

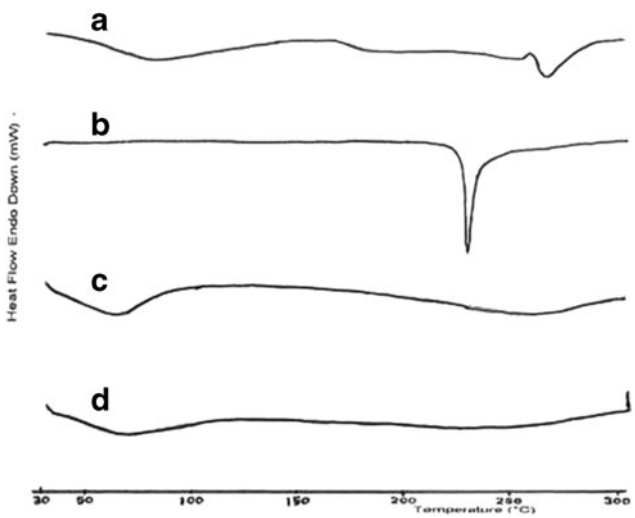


Fig. 4 DSC thermograms of CAS (**a**), ALF (**b**), Unloaded CAS nanoparticles (F_0) (**c**), ALF-loaded CAS nanoparticles (F_3) (**d**).

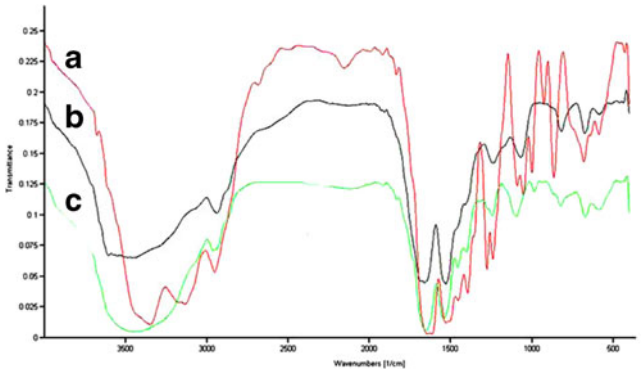


Fig. 5 FTIR spectra of ALF (**a**), CAS (**b**), ALF-loaded CAS nanoparticles (F_3) (**c**).

FTIR spectra of CAS, ALF and drug-loaded CAS nanoparticles were shown in Fig. 5. CAS typically shows absorption bands at 3455.86, 1661.12, 1530.51 and 1235.4 cm^{-1} that originate from N–H stretching and amide bending vibrations (38). CAS exhibited another characteristic band at 1415.9 cm^{-1}, attributable to the carboxylate group (O–C–O). The IR spectrum of ALF exhibited all the characteristic absorption bands of the functional groups of the drug. The bands at 3348.7, 3132.7 and 2952.2 cm^{-1} are characteristic of the primary and secondary amino groups of ALF whereas the bands at (1277.9 and 998.23 cm^{-1}) and (1238.9 and 867.26 cm^{-1}) are characteristic of the asymmetric and symmetric stretching vibrations of non-cyclic and cyclic ether (C–O–C), respectively.

The IR spectrum of the ALF-loaded CAS nanoparticles showed peaks at 984.07, 867.26, 1274.3 and 1242.5 cm^{-1} corresponding to those of drug. The peaks characteristic of the amino groups of drug seem to be interfered with the broad peak of CAS amino groups at 3455.86 cm^{-1}. The characteristic peak at 1415.9 cm^{-1}, attributable to the carboxylate group of CAS was shifted to a lower frequency of 1394.7 cm^{-1} in the spectrum of nanoparticles that may suggest ionic interaction between amino groups of drug and carboxylate group of CAS.

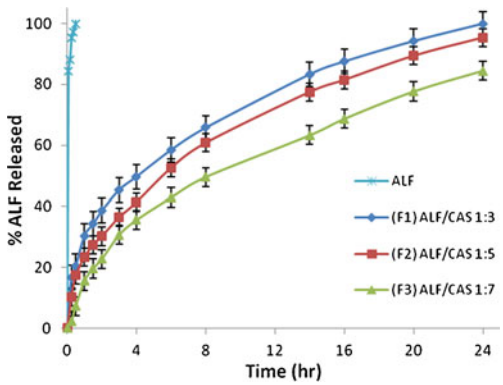


Fig. 6 Influence of the ALF/CAS mass ratio on the release behavior of ALF from spray-dried CAS nanoparticles in PBS pH 7.4 at 37°C.

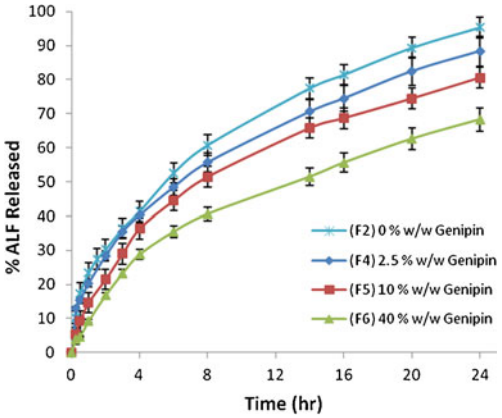


Fig. 7 Influence of the genipin concentration on the release behavior of ALF from spray-dried CAS nanoparticles in PBS pH 7.4 at 37°C.

The IR spectrum of genipin-crosslinked nanoparticles showed no spectral changes from the uncrosslinked nanoparticles (Data not shown).

In Vitro Drug Release

The ALF release from spray-dried CAS nanoparticles in PBS (pH 7.4) at 37°C was shown in Figs. 6, 7 and 8. The free drug showed a fast dissolution and reached about 100% of the initial dose after only few minutes characteristic of drugs with high water solubility (Fig. 6). The CAS nanoparticles containing ALF delayed drug dissolution when tested under the same conditions with no significant burst effect. The drug retention in the nanoparticles indicates that the drug is entrapped in the core of nanoparticles which provides a tremendous advantage for sustained release and long-term storage of our nanoparticles. Similar findings were reported by Amorim *et al.* (11), for idebenone release from spray-dried chitosan-TPP nanoparticles.

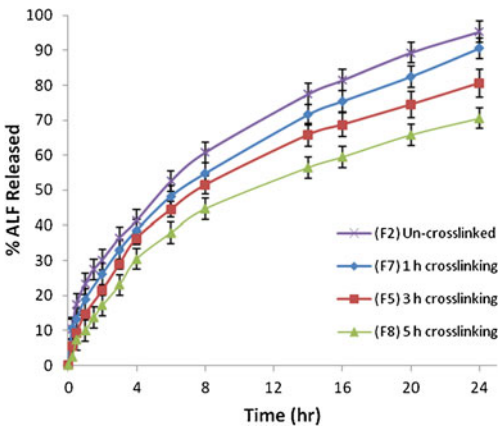


Fig. 8 Influence of the genipin crosslinking time on the release behavior of ALF from spray-dried CAS nanoparticles in PBS pH 7.4 at 37°C.

Table II Release Kinetic Parameters of ALF-loaded CAS Nanoparticles in PBS (pH 7.4) at 37°C

Formula	Variable	Zero Order	First Order	Higuchi	Korsmeyer-Peppas	
		R^2	R^2	R^2	R^2	n
	ALF/CAS mass ratio[a]					
F_1	1:3	0.880198	0.753188	0.988998	0.994255	0.45190
F_2	1:5	0.911811	0.983255	0.996767	0.989985	0.48093
F_3	1:7	0.921896	0.989738	0.995375	0.940397	0.82169
	Genipin conc.[b] (% *w/w*)					
F_4	2.5	0.909271	0.992471	0.997251	0.994540	0.45814
F_5	10	0.903750	0.986085	0.993102	0.997483	0.64888
F_6	40	0.921586	0.980246	0.993913	0.982601	0.68872
	Genipin crosslinking time (hr)[c]					
F_7	1	0.921416	0.989332	0.998687	0.996537	0.48911
F_5	3	0.903750	0.986085	0.993102	0.997483	0.64888
F_8	5	0.915406	0.978528	0.991354	0.972856	0.71820

[a] F_0-F_3 nanoparticles that are not crosslinked with genipin

[b] F_4, F_5 and F_6 were prepared using 1:5 ALF/CAS mass ratio and 3 h genipin-crosslinking time

[c] F_7, F_5 and F_8 were prepared using 1:5 ALF/CAS mass ratio and 10% *w/w* genipin

Figure 6 illustrates the influence of drug/polymer ratio on the drug release performance from un-crosslinked CAS nanoparticles. The cumulative ALF release from the CAS nanoparticles was observed to increase with the increase of ALF/CAS mass ratio used for its preparation. After the first 4 h, the cumulative ALF released was found to be 35.56, 41.34 and 49.68% from CAS nanoparticles prepared with 1:7, 1:5 and 1:3 ALF/CAS ratios, respectively. As the drug loading increased, this can lead to the enhancement of the driving force for diffusion inside the nanoparticles and outside in the release medium resulting in higher drug release rates (39).

ALF release was less from genipin-crosslinked CAS nanoparticles than that from the un-crosslinked ones over the entire 24 h test period. After 24 h, the amount of ALF released from un-crosslinked CAS nanoparticles was 95.32% while that from 2.5% *w/w* genipin-crosslinked ones was 88.34% (Fig. 7). By increasing the concentration of genipin as a chemical crosslinker to 10 and 40% (*w/w*), the release of ALF after 24 h was decreased to 80.56 and 68.23%, respectively (Fig. 7). The reason may be that at the higher concentration, more genipin was available to react with the amino groups of CAS resulting in an increased degree of protein crosslinking with a less available free space for drug diffusion. This denser nanoparticle matrix might also exhibit slower protein degradation, and hence slower drug release rates (40). Figure 8. shows that the release of ALF from genipin-crosslinked CAS nanoparticles decreased with increasing the crosslinking time. After 24 h, about 90.55, 80.56 and 70.55% of ALF was released from nanoparticles crosslinked with genipin for 1,3 and 5 h, respectively. This may be attributed to reduced swelling and erosion of CAS nanoparticles with the increased crosslinking density of the matrices (40).

Similar behavior was also reported for genipin-crosslinked CAS hydrogel where the release behavior of bovine serum albumin from the hydrogel could be related to various crosslinking and swelling degrees of the hydrogel networks formed by various amounts of genipin (24). In another study, Imsombut *et al.* (33), found that the genipin crosslinking can reduce the dissolution of silk fibroin microspheres in water. The % dissolution decreased by increasing of genipin ratio and crosslinking time suggesting that the degree of crosslinking of the microspheres increased with the genipin ratio or crosslinking time. The storage of the nanoformulations for 6 months at 25°C in tightly-closed vials in a desiccator was found to maintain their nanostructure. The particle size and the drug release profiles were not significantly affected. The nanoparticles were still redispersible in aqueous medium.

Release kinetics were evaluated by fitting the obtained release data into first order, zero order and Higuchi equations. The goodness of fit for the prepared formulations ranked in the following order: Higuchi > first order > zero order (Table II). To understand the drug release mechanism, the results were further analyzed according to the Korsmeyer–Peppas model. It is clear that the calculated n values are characteristic for the non-Fickian type of drug diffusion ($0.45 < n < 0.89$). These results indicate that the release of ALF from nanoparticles seemed to be controlled by coupled diffusion/polymer relaxation, indicating anomalous behavior which means that the processes of diffusion

and swelling run at comparable rates (3,25). . A substatial swelling of genipin-crosslinked CAS hydrogels in PBS (pH 7.4) solution was previously reported (24).

CONCLUSIONS

The preparation of dry solid form of casein nanoparticles for prolonged release of alfuzosin hydrochloride by the spray-drying process was investigated experimentally. The results demonstrated the potentiality of the technique for this application without using drying auxiliaries. Focusing on the size distribution after reconstitution, the preparations were easily reconstituted with the particle size of the redispersed particles was in the nano-range. Using genipin as a natural crosslinker of casein nanoparticles, a sustained drug release was obtained with the % drug released could be monitored *via* modulating the crosslinking density. This study supports the approach of using spray-drying technique to prepare redispersible genipin-crosslinked casein nanoparticles in a powdered form for prolonged drug release using casein itself as a spray-drying aid.

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