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Development and In Vitro Evaluation of Alginate Gel–Encapsulated, Chitosan-Coated Ceramic Nanocores for Oral Delivery of Enzyme

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The successful administration of protein and peptide drugs by oral route maintaining their active conformation remains a key challenge in the field of pharmaceutical technology. In the present study, we propose the use of a nanosize ceramic core-based system for effective oral delivery of acid-labile model enzyme, serratiopeptidase (STP). Ceramic core was prepared by colloidal precipitation and sonication of disodium hydrogen phosphate solution and calcium chloride solution at room temperature. The core was coated with chitosan under constant stirring and Fourier-Transform Infra Red Spectroscopy (FTIR) confirmed phosphoric groups of calcium phosphate linked with ammonium groups of chitosan in the nanoparticles; then the enzyme was adsorbed over the preformed nanocore. Protein-loaded nanocore was further encapsulated into alginate gel for enzyme protection. Prepared system was characterized for size, shape, loading efficiency, and in vitro release profile (pH 1.2 and pH 7.4). The effect of processing variables on the size of the core was evaluated to form small, uniform, and discrete nanocores. Stability and integrity of enzyme during processing steps was assessed by in vitro proteolytic activity. The prepared system was examined to be spherical in shape with diameter 925 ± 6.81 nm using TEM. The in vitro release data followed the Higuchi model, showing a low amount (26% ± 2.4%) of diffusion-controlled drug release (R^2 = 0.9429) in acidic buffer up to a period of 2 to 6 hours, signifying the integrity of alginate gel in acid. In the alkaline medium sustained and nearly complete first order release of protein was observed up to a 6 hours. It is inferred that the protein-loaded ceramic core acts as a reservoir of the adsorbed enzyme and alginate gel provides protection to STP for controlled release in intestinal pH when compared to the enzyme solution.

Keywords ceramic nanocore; chitosan; serratiopeptidase; oral delivery; proteolytic activity; alginate; peptide

INTRODUCTION

Recent advancements in biotechnology and genetic research have led to an increased surge of interest in the use of peptide and protein drugs (Zhou & Li, 1991b). But many of them require special formulation technologies to overcome drug-associated problems such as chemical and physical instability, poor bioavailability, and potentially strong side effects requiring drug enrichment at the site of action.

Proteolytic enzymes represent an important class of protein and peptides with primary pharmacological use as anti-inflammatory and digestive agents. One of the clearest indications of the general recognition of this premise is the vast annual expenditure of the pharmaceutical industry on exploring the involvement of peptidases in human health and disease (Barret, 1991). Among this category, serratiopeptidase (STP) offers a powerful treatment for pain and inflammation with widespread use in arthritis, fibrocystic breast disease, chronic bronchitis, and carpal tunnel syndrome (Kee, Tan, Lee, & Salmon, 1989; Majima, 1990).

These are normally delivered by the parenteral administration. However complications such as thromboflebitis or tissue necrosis and poor patient compliance have stimulated the investigation of non-parenteral routes (Zhou, 1994). Among non-parenteral routes, oral administration is usually preferred because it is most acceptable and convenient for the patient. But oral bioavailability of these peptide drugs is generally very low, owing to the acidic conditions of the stomach, proteolytic activity of the gastrointestinal tract, and poor permeability across intestinal mucosa (Zhou & Li, 1991a). Various approaches have been proposed to overcome the biopharmaceutical limitations associated with these drugs, such as inhibition of the enzymatic degradation (Morimoto et al., 1991), chemical modification of the protein (Conradi, Hilghers, Ho, & Burton, 1992), in situ gel system (Shah & Paradkar, 2005), and the formulation of polymer-based carrier systems (Torchilin, Tischenko, Smirnov, & Chazov, 1977).

Application of nanocarriers for drug delivery, especially bioactive drugs, is an expanding area of research (Rawat, Singh, Saraf, & Saraf, 2006) that provided the design of biomaterials with controlled rates of drug release (Prokop, Kozlov, Newman, & Newman, 2002). It is tempting to expand the

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utility of nanocarriers for delivery of therapeutic enzymes, because particles larger than 20 μm are prone to be washed out, being inefficient for mucosal delivery (Lameiro, Lopes, Martins, Alves, & Meloa, 2006).

But formulation processes must maintain the native three-dimensional structure and chemical integrity of the native protein upon administration (Cleland, 1997). Thus the greatest limiting factor with these fragile modules as clinical tools is due to the interaction between the drug carrier and the drug, which affects physiochemical stability. Innovative techniques have led to the use of ceramics in high-tech applications. These ceramic systems are capable of delivering chemicals and biologicals effectively while maintaining their structural conformation and activity (Cherian, Rana, & Jain, 2000; Kossovsky, 1990).

The aim of the present study is to design chitosan-coated nanocores (CNC) for effective loading and protection of an active acid-labile large enzyme, STP. As an attempt towards increasing the bioavailability, conformational stability, and activity of the enzyme, we have loaded STP into chitosan-coated ceramic nanocores and encapsulated them into alginate gel for effective protection, mucoadhesiveness, and sustained release. In protein carrier selection, natural polymers such as chitosan and alginate were selected because they are biosafe; highly inert towards protein drugs; do not need organic solvents; and possess properties of mucoadhesiveness, biodegradability, low toxicity, low immunogenicity, ready availability, and inexpensiveness (Gombotz & Wee, 1998). In particular, morphology, loading, in vitro proteolytic activity, and release profile of prepared nanocarriers were evaluated as a function of the preparation procedure.

MATERIALS AND METHODS

Materials

STP (MW 52kDa) and chitosan (with 80% degree of deacetylation and viscosity of 16 mPa) were received as gift samples from Advanced Enzyme Technologies Ltd., Nasik, India, and Chemchito Natural Products, Chennai, India. Sodium alginate and calcium chloride dihydrate were purchased from Loba Chemicals, Mumbai, India, and S. D. Fine Chemicals Ltd., Mumbai, India. Calcium phosphate was synthesized in the laboratory. All other chemicals were of analytical grade.

Preparation of Alginate Gel–Encapsulated, Chitosan-Coated Nanocores (ACNC)

Ceramic cores made up of calcium phosphate dihydrate were prepared in the laboratory by a procedure reported by Kossovsky and colleagues (1990) with slight modifications. A 0.90 M solution of Na_2HPO_4 was slowly added to the 0.30 M solution of calcium chloride and was sonicated (10 W) for 2 hours at 4°C. Precipitate of calcium phosphate was separated by centrifugation at 7,200 g for 1 hour and then washed five times with 50 mL distilled water to remove even traces of sodium chloride formed during the reaction. The precipitate was resuspended in distilled water and passed through a 0.2-μm millipore filter to collect particles less than 0.2 μm. Obtained calcium phosphate dihydrate core particles were dispersed in chitosan solution (0.1% to 0.6% w/v) for effective coating under constant stirring and lyophilization. Three percent (3%) w/v sodium tripolyphosphate (STPP) solution was added dropwise to the system as a cross-linking agent. Various process parameters such as core to coat ratio (1:1 to 1:5), chitosan concentration (0.1% to 0.6% w/v), stirring speed (3,000 to 12,000 g), and time (20 to 120 minutes) were optimized to get small, spherical, discrete coated nanoparticles. Obtained chitosan-coated ceramic nanocores were washed several times with distilled water. Proteolytic enzyme STP was loaded into these CNC by soaking these particles in a fixed amount of STP solution (0.1% w/v solution of STP in saline phosphate buffer, pH 7.4) for 24 hours by constant stirring. These enzyme-loaded nanocarriers were mixed with sodium alginate solution (2% w/v). To this, $CaCl_2$ solution was added with Ca/alginate mass ratio 0.6% w/w for complete alginate gel formation (De & Robinson, 2003) and stirred for 30 minutes to obtain particles encapsulated in alginate gel. All operations were carried out at temperature below 4°C.

Morphology and Structural Characterization

The average size and size distribution of plain, protein-adsorbed chitosan-coated ceramic nanocores and alginate gel–encapsulated system were determined by transmission electron microscopy (TEM, CM-12 Philips, Netherlands) after negative staining with 1% phosphotungstic acid. Chitosan coating on CNC was ascertained by FTIR. CNC were separated from the suspension and dried by a freeze dryer (HETO Power DryLL3000, Denmark), and their FTIR (Schimadzu FTIR-8400S, Tokyo, Japan) was taken with KBr pellets.

Enzyme Loading Efficiency

The amount of enzyme loaded onto calcium phosphate dihydrate core was determined by a method reported by Loukas and Gregoriadis (1997). Control formulations (without protein coating) were incubated under constant stirring with the known concentration of drug for 24 hours at 4°C. The supernatant was separated after centrifugation at 9,000 g for 1 hour below 4°C in a refrigerated centrifuge (IV C1-6363, Remi Instruments, Mumbai, India). The protein remaining in the supernatant liquid after loading was estimated by measuring absorbance at 229.5 nm by first derivative method spectrophotometerically (Shimadzu UV-1700, Pharmaspec, Tokyo, Japan).

In Vitro Release Studies

In order to visualize the release profile of the oral drug delivery system, in vitro release profile of enzyme was carried

out both in HCl (pH 1.2) and Phosphate Buffer Saline (PBS; pH 7.4) buffer. One hundred (100) mg of enzyme-loaded carriers were introduced into 10 mL of respective medium under magnetic stirring (100 rpm) maintained at a temperature of 37° ± 0.5°C. At various time intervals, 0.1 mL of sample was withdrawn and replenished with fresh dissolution medium maintained at 37° ± 0.5°C. Samples withdrawn from acidic medium were centrifuged at 9,000 g for 10 minutes and the supernatant was used for protein analysis. Samples obtained from phosphate buffer were mixed with 0.2 M NaOH to raise pH above 7.0; then ethanol was added (50/50, v/v) to precipitate alginate. Samples were then centrifuged and analyzed similarly to the sample obtained at pH 1.2.

In Vitro Proteolytic Activity

Prepared ACNC and plain STP solution were placed separately in HCl buffer (pH 1.2) or phosphate buffer (pH 7.4) maintained at 37° ± 0.5°C and stirred constantly at 100 rpm. After 2 hours, protein was recovered as reported previously for release studies. Samples were then assayed for proteolytic activity ($n = 3$).

The proteolytic activity was determined as per the method reported in Food Chemical Codex (2003). The assay was based on a 30-minute proteolytic hydrolysis of casein at 37°C and pH 7.0. Unhydrolyzed casein was removed by filtration and the solubilized casein was determined spectrophotometrically at a wavelength of 275 nm. In this method, the protease activity is expressed as protease unit (PC) units of preparation derived from *Bacillus subtilis* var. and *Bacillus licheniformis* var. One bacterial protease unit (PC) is defined as quantity of enzyme that produces 1.5 µg/mL equivalent of L-tyrosine per minute under the condition of the assay.

Activity of enzyme was calculated by equation:

$$\frac{PC}{g} = \left(\frac{A_u}{A_s}\right)\left(\frac{0.08}{30w}\right)$$

A_u is the value obtained by subtracting blank reading from test reading; A_s the absorption of standard solution; 0.08 the final volume in mL of reaction mixture; 30 the time of the reaction in minutes; and w the weight of the original sample in g.

Statistical Analysis

The results were expressed as mean ± standard deviation. Statistical analysis was carried out by student t-test, and statistical significance was designated as $p < .05$ (SPSS).

RESULTS AND DISCUSSION

The calcium phosphate dihydrate ceramic nanocores (NC) were prepared by slight modification of reported method (Kossovsky, 1990). During sonication, the nanosized NC self-assemble due to increase in the surface free energy of calcium phosphate particles (Kossovsky et al., 1996).

Ceramic cores were coated with the thin film of chitosan solution (1% w/v acetic acid) by stirring at different speeds with 3% w/v STPP as cross-linking agent. STPP (3% w/v) was selected as it is a nontoxic polyanion capable of forming cross-linked networks with chitosan. It specifically interacts with positively charged amine residues of chitosan with the degree of cross-linking dependent on the concentration of STPP (Taqieddin, Lee, & Amiji, 2002).

Various process variables, for example, chitosan concentration, core-to coat ratio, stirring speed, and stirring time, were studied to obtain uniformly sized spherical discrete CNC (Table 1). Chitosan was used in the concentration ranging from 0.1% to 0.6% w/v with fixed core concentration (1% w/v). The size of CNC increased with the increase in the chitosan concentration up to 0.5% w/v. Kawashima, Yamamoto, Takeuchi,

TABLE 1
Effect of Process Parameters on the Particle Size of the CNC

S. No.		Observation	Size (nm)
a) Core-to-Coat Ratio			
1.	1:1	Irregular	162 ± 1.65
2.	1:2	Irregular	274 ± 1.43
3.	1:3	Non-uniform spherical	396 ± 6.54
4.	1:4*	Spherical	410 ± 4.32
5.	1:5	Spherical	412 ± 4.34
b) Stirring Time (minutes)			
1.	20	Large irregular	784 ± 4.42
2.	40	Large discrete	718 ± 4.23
3.	60	Moderately discrete	645 ± 2.31
4.	80	Small spherical	542 ± 3.12
5.	100*	Discrete small spherical	419 ± 3.26
6.	120	Aggregates	684 ± 3.24
c) Stirring Speed (g)			
1.	3,000	Large discrete	982 ± 6.78
2.	6,000	Comparatively small	762 ± 8.84
3.	9,000*	Small spherical	412 ± 3.58
4.	12,000	Distorted spherical aggregates	678 ± 6.89

Chitosan concentration used was 0.5% w/v. Values are shown as representative of $M \pm SD$ for three independent determinations ($p < .05$). *variables selected for study.

and Kuno (2000) also supported the same result. Chitosan concentrations above 0.5% w/v, for example, 0.6% w/v, resulted in aggregated mass, which may be due to increased viscosity of chitosan solution. So the concentration of chitosan selected was 0.5% w/v. The effect of core-to-coat ratio from 1:1 to 1:5 on the size of CNC showed that the size of the nanocores increased from 162 ± 1.65 nm to 412 ± 4.34 nm (Figure 1 and Table 1). The size of the coated particles increased up to 1:4 core-to-coat ratio, and then no significant increase in size was observed, which is in agreement with results of Cherian and colleagues (2000). Moreover, highly aggregated forms were observed at this concentration, which could be due to the saturation of the free surfaces of the core with the coating material. Within the range of stirring time (20 to 120 minutes) investigated, the size and shape of the CNC changed from irregular large particles (784 ± 4.42 nm) to comparatively small uniform particles (419 ± 3.26 nm); at 120 minutes, larger particles were observed, which might be due to aggregation of small particles leading to increase in size (Figure 2). Similarly, the size decreased significantly from 982 ± 6.78 nm to 412 ± 3.58 nm on increasing the speed of stirrer from 3,000 to 9,000 g ($p < .05$). But on further increasing the speed to 12,000 g up to 100 minutes, increase in size with aggregated forms was observed. This may be due to increase in free surface energy, which increased the tendency of small particles to aggregate (Figure 3). Therefore, a 1:4 core-to-coat ratio, 100 minutes stirring time, and 9,000 g as stirring speed were selected to get spherical, chitosan-coated, discrete nanoparticles, as can be seen through TEM photographs (Figure 4).

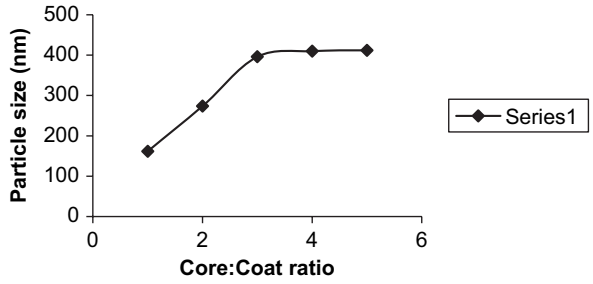

FIGURE 1. Effect of core-to-coat ratio on the size of prepared CNC. Results are given as $M \pm SD$ ($p < .05$).

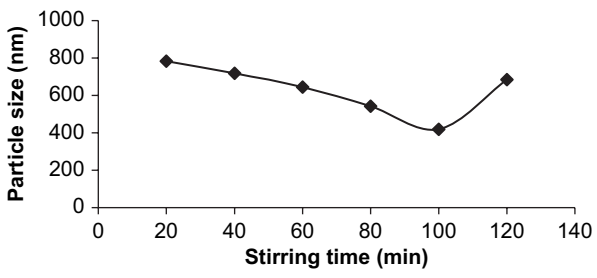

FIGURE 2. Effect of stirring time on the size of prepared CNC. Results are given as $M \pm SD$ ($p < .05$)

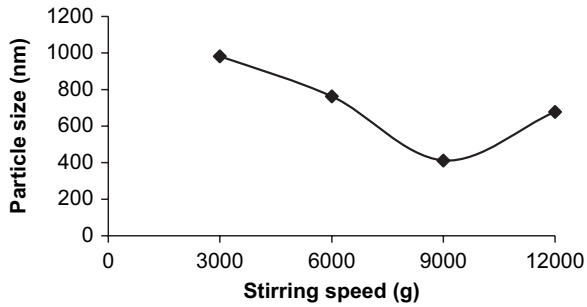

FIGURE 3. Effect of stirring speed on the size of CNC. Results are given as $M \pm SD$ ($p < .05$).

Chitosan coating on the ceramic cores could not be clearly identified in the TEM images. This could be due to close and longitudinal attachment of the CS chains during process required for TEM visualization (Garcia-Fuentes, Torres, & Alonso, 2005). CS coating results in 100% increase in the size of ceramic cores due to the deposition of CS layers on the surface of the nanocores.

FTIR spectra of chitosan-coated calcium phosphate nanocores (CNC) and chitosan are shown in Figure 5. FTIR spectra of chitosan represents characteristic band at 3,434/cm due to $–NH_2$ and –OH group stretching vibration in chitosan matrix. In CNC a shift in peak from 3,434/cm to 3,399/cm was observed, previously attributed to enhanced hydrogen bonding (Yu, Du & Zheng, 1999). In CNC the peak at 1,644/cm disappeared and a new sharp peak at 1,632/cm appeared, and the 1,604/cm peak of $–NH_2$ bending vibration shifted to 1,536/cm. This shift in peak of $–NH_2$ group can be due to the linkage between phosphoric group of calcium phosphate and ammonium ion of chitosan. A similar result was obtained in the study of chitosan film treated with phosphate NaH_2PO_4 (Knaul, Hudson, & Creber, 1999). Chitosan exhibits good film-forming abilities and stabilized the core through ionic and noncovalent forces. TEM results and FTIR studies provided a clear evidence of the chitosan coating due to increase in particle size and shift in the peak heights, respectively.

After the optimization of processing variables (0.5% w/v chitosan concentration, 1:4 core-to-coat ratio, 100-minute stirring time, 9,000 g stirring speed) on the basis of size and shape of CNC, the enzyme was allowed to adsorb on the CNC by a partial adsorption technique. The increase in the size of nanocores was evident from the TEM study of STP-loaded CNC (524 ± 4.46 nm) (Figure 4c; Table 2). Chitosan is soluble in a weak acidic solution, and thus the use of organic solvent can be avoided, which is favorable for maintaining bioactivity of protein and peptide drugs. Moreover, the amino groups of chitosan are protonated in an acidic solution and the resultant soluble

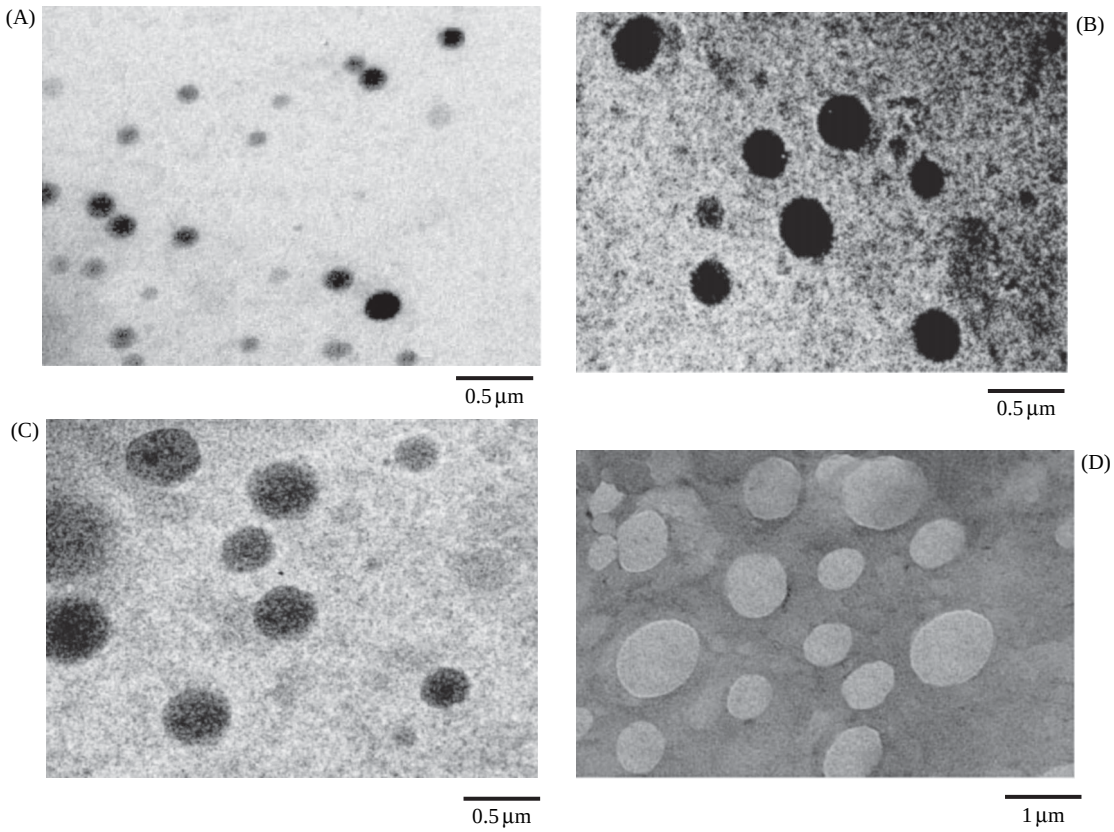

FIGURE 4. TEM of various stages of nanocarriers; (A) Calcium phosphate nanocores (NC); (B) Chitosan-coated nanocores (CNC); (C) STP-adsorbed CNC; (D) Alginate-encapsulated CNC (ACNC).

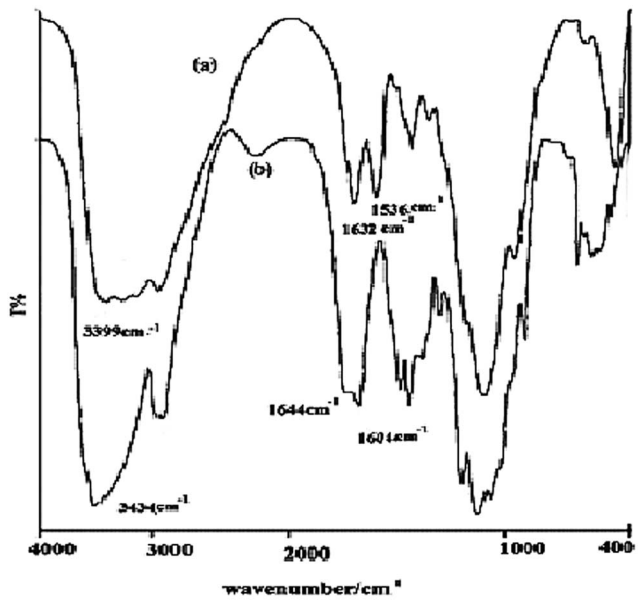

FIGURE 5. FTIR spectra of (A) chitosan-coated calcium phosphate nanocores and (B) pure chitosan. FTIR spectra indicate the clear shift in the peak height around 1,604/cm to 1,536/cm, indicating the linkage of phosphoric group of calcium phosphate with ammonium group of chitosan.

polysaccharide is positively charged, which can bind strongly to negatively charged surfaces of proteins above its isoelectric point (Janes, Calvo, & Alonso, 2001). Iso-electric point of the STP obtained from *Serratio marcescens* is 6.1 and above this pH it attains negative charge, which is suitable for its binding with the positively charged chitosan surface (Salamone & Wodzinski, 1997). These surface-adsorbed nanocores provide conformational stabilization as well as a high degree of surface exposure to proteins (Kossovsky et al., 1996; Paul & Sharma, 2001).

Enzyme-loaded CNC were encapsulated in alginate gel for protection of STP from getting exposed to acidic pH in the stomach (Coppi, Iannuccelli, Leo, Bernabei, & Cameroni, 2002). Alginate gel encapsulation resulted in an increase in the size of ACNC from 524 ± 4.46 nm to 925 ± 6.81 nm.

The results of size analysis data for the various stages of nanocarriers measured by TEM are given in Table 2. With each additional coating of ceramic cores with the chitosan, enzyme, and alginate consecutively resulted in the increase in size of nanocores, which is also evident from the TEM photographs (Figure 4).

Enzyme loading efficiency of CNC (size 415 ± 4.26 nm) was determined and found to be 46.64% ± 1.48%. The size of STP-adsorbed CNC increased about 580 ± 4.46 nm due to

TABLE 2
Particle Size of Different Stages of Nanocores with Net Protein Loading

S. No.	Core Size (nm)	CNC	Enzyme-Adsorbed Ceramic Core Size (nm)	Alginate-Encapsulated System (nm)	Enzyme Loading (%)
1.	142 ± 4.26	415 ± 4.26	524 ± 4.46	925 ± 6.81	46.64 ± 3.48

All the values are representative of $M \pm SD$ for three independent determinations ($p < .05$).

adsorption of drug molecules on the surface of cores coated with 0.5% w/v chitosan solution. The increased protein payload with CS coated nanocores was observed due to the increase in size.

Proteolytic activity of alginate-encapsulated nanocarriers was evaluated separately before and after treating them for 2 hours in acidic (0.1N HCl, pH 1.2) and basic media (phosphate buffer, pH 7.4); results are presented in Figure 6. The nanocarriers showed about 4.35% ± 0.08% loss of proteolytic activity in acidic medium whereas retention of activity in basic medium was found to be 98.26% ± 0.32%. Meager loss of activity in alkaline media may be due to processing steps involved in preparation of formulation. At the same time plain STP solution exhibited almost complete loss of activity in acidic medium and 86.84% activity was retained in alkaline medium. ACNC exhibited much better retention of proteolytic activity compared with plain STP solution. Possible explanation for the improved physical and chemical stability of proteolytic enzyme in nanocarriers may be due to reduced mobilization of protein due to ionic association with the positively charged polymer, that is, chitosan and alginate gel coating.

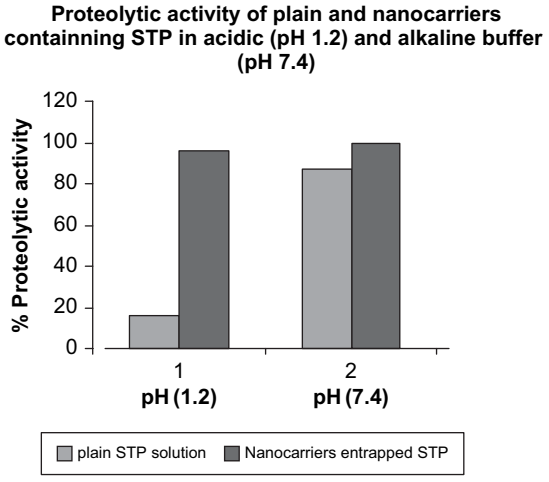

FIGURE 6. Effect of acidic and alkaline pH on the in vitro proteolytic activity of plain STP solution and STP entrapped in ACNC. Proteolytic activity was determined on the basis of 30-minute proteolytic hydrolysis of casein at 37°C and pH 7.0 as reported in food chemical codex (2003). Values are shown as mean of three independent determinations.

The transit time of a drug through the absorptive area of the gastrointestinal tract (GIT) is between 9 and 12 hours (Gibaldi & Perrier, 1982), whereas γ scintigraphy studies confirm a short GIT transit time from mouth to cecum of 4 to 6 hours (Shargel & Yu, 1999). Thus assuming a maximum GIT transit time of 12 hours, a formulation in the cecum is expected to release its drug load within 6 hours. Considering the same, in vitro drug release from the ACNC was studied for duration of 6 hours. Release of encapsulated protein in HCl buffer pH 1.2 was found to be significantly less than in PBS (pH 7.4). 99.6% ± 0.2% of the drug was released in PBS during a period of 6 hours (Figure 7). However, during the same period only 26.58% ± 2.4% of the drug was released in HCl buffer. Release seems to be spontaneous in the alkaline pH, unlike alginate-encapsulated nanoparticles.

In order to investigate the release mechanism of present drug delivery system, the release data of prepared ACNC in acidic (pH 1.2) and alkaline (pH 7.4) buffer were fitted to classic drug release kinetics models. The release rates were analyzed by least square linear regression method. Release models such as first order model, Higuchi model, and Ritger-Peppas empirical model were applied to the release data (Dredan, Antal, & Racz, 1996; Peppas, 1985) (Table 3). The coefficient of determination (R^2) of equation for release of STP from ACNC in alkaline buffer was 0.9664, whereas in acidic buffer it was 0.6502, signifying first order release pattern followed by ACNC in the alkaline medium. There was an initial burst release of 15.6% ± 0.24% and 25% ± 0.46% in the first hour in both acidic and alkaline buffer, respectively. So the release

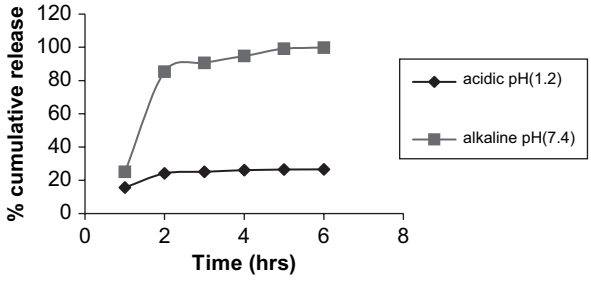

FIGURE 7. In vitro release profile of STP from optimized ACNC in acidic pH (1.2) and alkaline pH (7.4) buffer. Results are given as $M \pm SD$ ($p < .05$).

TABLE 3
Release Behavior of STP in Acidic and Alkaline Buffer

S. No.	Kinetics		ANC pH 1.2	ANC pH 7.4
1.	First order			
		K	0.02303	0.9481
		R^2	0.6502	0.9664
2.	Higuchi			
	(1–6 hr)	K	6.7338	44.87
		R^2	0.7394	0.6925
	(2–6 hr)	K	2.4378	12.796
		R^2	0.9429	0.8609
3.	Ritger-Peppas			
	(1–6 hr)	n	0.2789	0.7123
		R^2	0.8088	0.7438
	(2–6 hr)	n	0.0916	0.1346
		R^2	0.9653	0.9026

K, release rate constant; R^2, coefficient of determination; n, release exponent.

data after 1 hour were fitted to Higuchi and Ritger-Peppas equation. The value of coefficient of determination (R^2) in Higuchi equation was found to be 0.9429 and 0.8609 in acidic and alkaline buffer medium, respectively, which indicates the integrity of alginate gel and diffusion-controlled release between 2 and 6 hours in acidic buffer, whereas in alkaline buffer, R^2 value doesn't favor diffusion pattern. This difference in release pattern is due to pH sensitivity of alginate gel. At pH 1.2, calcium alginate gel is converted to a unionized form of alginic acid. Even if Ca^{+2} ions don't contribute any more to stabilization of the system leading to initial burst, alginate system maintains its structure without any visible changes in morphology (Zhou, Deng, & Li, 2001). This insight leads to dissociation of ionic linkages and reduction in gel strength that may favor drug release by diffusion (Ostberg, Lund, & Graffner, 1994). All this is responsible for decreased release of protein in acidic pH even after 2 hours. Substituting the release values determined between 2 and 6 hours in the Ritger-Peppas equation, the value of coefficient of determination was about 0.9 in each case. But the value of n obtained was confusing as value of $n = 0.43$ indicates Fickian (case I) release; > 0.43 but < 0.85 for non-Fickian (anomalous) release; and > 0.89 indicates super case II type of release. Case II generally refers to the erosion of the polymeric chain and anomalous transport (non-Fickian) refers to a combination of both diffusion and erosion controlled–drug release (Siepmann & Peppas, 2001). The results showed that the Ritger-Peppas model was not suitable for estimating the release kinetics of ACNC. It was believed to be due to the immediate release of STP caused by rapid dissolution of the alginate in the alkaline buffer. At higher pH, alginic acid is converted to sodium salt of alginate and the matrix gets disintegrated completely, releasing the entrapped core. But because of the protein-adsorbed core structure, whole protein is not released as a burst maintaining sustained effect. This result was attributable to the slight sustained release of drug signifying mixed type of release pattern.

CONCLUSION

ACNC appeared to be a promising system for the association and delivery of sensitive macromolecules such as proteins and peptides due to mild condition requirements for their formation. The prolonged activity was obtained due to slow release of the enzyme from the ACNC and the intact structure without denaturation or dehydration during delivery and storage. The encouraging results obtained in this study could propose ACNC for future in vivo studies, especially in the delivery of protein and peptide drugs. These novel nanocarriers were found to be promising for protection of the spatial qualities for exhibiting better therapeutic effect. But further studies in terms of pharmacokinetics, toxicology, and animal studies are required for clinical utility of the formulation.

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REFERENCES

Barret, A. (1991). Peptidases: A view of classification and nomenclature. In: V. Turk (Ed.), *Proteases: A new perspective* (pp. 1–56). Basel, Switzerland: Birkhauser Verlag.

Cherian, A. K., Rana, A. C., & Jain, S. K. (2000). Self-assembled carbohydrate-stabilized ceramic nanoparticles for the parenteral delivery of insulin. *Drug. Dev. Ind. Pharm.*, *26*(4), 459–463.

Cleland, J. L. (1977). Protein delivery from biodegradable microspheres. In: L. M Sander & R. W. Hendren (Eds.), *Protein delivery: Physical systems* (pp. 1–43). New York: Plenum Press.

Conradi, R. A., Hilghers, A. R., Ho, N. F. H., & Burton, P. S. (1992). The influence of peptide structure on transport across Caco-2 cells: II. Peptide bond modification, which results in improved permeability. *Pharm. Res*., *9*, 435–439.

Coppi, G., Iannuccelli, V., Leo, E., Bernabei, M. T., & Cameroni, R. (2002). Protein immobilization in crosslinked alginate microparticles, *J. Microencapsulation*, *19*(1), 37–44.

De, S., & Robinson, D. (2003). Polymer relationships during preparation of chitosan- alginate and poly-l-lysine–alginate nanospheres. *J. Control. Release*, *89*, 101–112.

Dredan, J., Antal, I., & Racz, I. (1996). Evaluation of mathematical models describing drug release from lipophilic matrices. *Int. J. Pharm*., *145*, 61–64.

Garcia-Fuentes, M., Torres, D., & Alonso, M. J. (2005). New surface modified lipid nanoparticles as delivery vehicles for salmon calcitonin. *Int. J. Pharm*., *296*, 122–132.

Gibaldi, M., & Perrier, D. (1982). *Pharmacokinetics* (2nd ed.). New York: Marcel Dekker.

Gombotz, W. R., & Wee, S. F. (1998). Protein release from alginate matrices. *Adv. Drug Del. Rev.*, *31*, 267–285.

Janes, K. A., Calvo, P., & Alonso, M. J. (2001). Polysaccharide colloidal particles as delivery systems for macromolecules. *Adv. Drug Delivery Rev*., *47*, 83–97.

Kawashima, Y., Yamamoto, H., Takeuchi, H., & Kuno, Y. (2000). Mucoadhesive DL-lactide/glycolide copolymer nanospheres coated with chitosan to improve oral delivery of calcitonin. *Pharmaceutic. Develop. Technol*., *5*, 77–85.

Kee, W. H., Tan, S. L., Lee, V., & Salmon, Y. M. (1989). The treatment of breast engorgement with serrapeptase: A randomized double blind controlled trial. *Singapore Med J*., *30*(1), 48–54.

Knaul, J. Z., Hudson, S. M., & Creber, K. A. M. (1999). Improved mechanical properties of chitosan fibers. *J. Appl. Polym. Sci*., *72*, 1721–1731.

Kossovsky, N., Bunshah, R. F., Gelman, A., Sponsler, E., Umarjee, D. M., Suh, T. G., et al. (1990). A non-denaturing solid phase pharmaceutical carrier comprised of surface-modified nanocrystalline materials. *J. Appl. Biomater.*, *1*, 289–294.

Kossovsky, N., Gelman, A., Rajguru, S., Nguyen, R., Sponsler, E., Hnatyszyn, H. J. et al. (1996). Control of molecular polymorphisms by a structured carbohydrate/ceramic delivery vehicle—Aquasomes. *J. Control. Release*, *39*, 383–388.

Lameiro, M. H., Lopes, A., Martins, L. O., Alves, P. M., & Meloa, E. (2006). Incorporation of a model protein into chitosan–bile salt microparticles. *Int. J. Pharm*., *312*, 119–130.

Loukas, Y. L., & Gregoriadis, G. (1997). Novel liposome based systems for the protection of labile drugs. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, *24*(104), 204.

Majima, Y., Hirata, K., Takeuchi, K., Hattori, M., & Sakakura, Y. (1990, January). Effects of orally administered drugs on dynamic viscoelasticity of human nasal mucus. *Am. Rev. Respir. Dis*., *141*(1), 79–83.

Morimoto, K., Yamaguchi, H., Iwakura, Y., Miyazaki, M., Nakatami, E., Iwamoto, T., et al. (1991). Effects of proteolytic enzyme inhibitors on the nasal absorption of vasopressin and an analogue. *Pharm. Res*., *8*, 1175–1179.

Ostberg, T., Lund, E. M., & Graffner, C. (1994). Calcium alginate matrices for oral multiple unit administration: IV release characteristics in different media. *Int. J. Pharm.*, *112*, 241–248.

Paul, W., & Sharma, C. P. (2001). Porous hydroxyapatite nanoparticles for intestinal delivery of insulin. *Trends Biomat. Art. Organs*, *14*(2), 37–38.

Peppas, N. A. (1985). Analysis of Fickian and non-Fickian drug release from polymers. *Pharm. Acta. Helv*., *60*, 110–111.

Prokop, A., Kozlov, E., Newman, G. W., & Newman, M. J. (2002). Water based nanoparticulate polymeric system for protein delivery: Permeability control and vaccine application, *Biotechnol. Bioeng*., *78*, 459–466.

Rawat, M., Singh, D., Saraf, S., & Saraf, S. (2006). Nanocarriers: Promising vehicle for bioactive drugs. *Biol. Pharm. Bull*., *29*(9), 1790–1798.

Salamone, P. R., & Wodzinski, R. J. (1997). Production, purification and characterization of a 50-kDa extracellular metalloprotease from *Serratia marcescens*. *Appl. Microbiol. Biotechnol*., *48*, 317–324.

Shah, M., & Paradkar, A. (2005). Cubic liquid crystalline glyceryl monooleate matrices for oral enzyme delivery. *Int. J. Pharm*., *294*, 161–171.

Shargel, L., & Yu, A. (1999). *Applied biopharmaceutics and pharmacokinetics* (4th ed.). London: Prentice Hall.

Siepmann, J., & Peppas, N. A. (2001). Modelling of drug release from delivery systems based on hydroxypropyl methyl cellulose (HPMC). *Adv. Drug Del. Rev*., *48*, 139–157.

Taqieddin, E., Lee, C., & Amiji, M. (2002). Permselective chitosan of hybrid microcapsules for enzyme immobilization. *Pharm. Eng*., *22*(6), 1–3.

Torchilin, V. P., Tischenko, E. G., Smirnov, V. N., & Chazov, E. I. (1977). Immobilization of enzymes on slowly soluble carriers. *J. Biomed. Mater. Res*., *11*, 223–235.

Yu, J. H., Du, Y. M., & Zheng, H. (1999). Blend films of chitosan*/gelation. *J. Wuhan Univ. (Nat. Sci. Ed.)*, *45*, 440–444.

Zhou, X. H. (1994). Overcoming enzymatic and absorption barriers to non-parenterally administered protein and peptide drugs. *J. Control. Release*, *29*, 239–252.

Zhou, S., Deng, X., & Li, X. (2001). Investigation on a novel core coated microspheres protein delivery system. *J. Control. Release*, *75*, 27–36.

Zhou, X. H., & Li, W. P. (1991a). Comparison of enzyme activities of tissues lining portals of absorption of drugs: Species differences. *Int. J. Pharm*., *70*, 271–283.

Zhou, X. H., & Li, W. P. (1991b). Peptide and protein drugs: I. Therapeutic applications, absorption and parenteral administration. *Int. J. Pharm*., *75*, 97–115.