Phthalyl Chitosan–Poly(ethylene oxide) Semi-Interpenetrating Polymer Network Microparticles for Oral Protein Delivery: An *In Vitro* Characterization

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ABSTRACT: Phthalyl chitosan (PC) was synthesized and phthalyl chitosan–poly(ethylene oxide) (PCP) semi-interpenetrating network microparticles were developed by ionic crosslinking with sodium tripolyphosphate. The characterization of PCP particles was done, and these particles were compared with PC and native chitosan (NC) microparticles. The PCP particle size was around 1.3 μm with a ζ potential of about -28.6 ± 12.6 mV and an insulin loading efficiency of 89.6%. The release studies were done at pH 1.2 and 7.4, which indicated a minimal release at pH 1.2 compared to that at pH 7.4. The degree of swelling was observed to be higher in PCP than in PC or NC particles. The *in vitro* mucin-binding capacity and the intestinal mucoadhesiveness of the particles were evaluated. The PCP particles were highly mucoadhesive, and correspondingly, the mucin-binding capacity was lower for these particles; this is necessary for any matrix to be a successful mucoadhesive. These results suggest the usefulness of these particles as a potential candidate for oral insulin-delivery systems. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 110: 2787–2795, 2008

Key words: chitosan; drug delivery systems; interpenetrating networks (IPN); protein

INTRODUCTION

Protein/peptide drugs have now become unavoidably important therapeutics with the progress in the areas of biochemistry, biopharmaceutics, and biotechnology. However, these drugs are administered parenterally for treatment. A noninvasive drug-delivery system for proteinaceous drugs still remains a goal to be achieved. Protein drugs are macromolecules, and they face mainly two hurdles for oral administration. Being proteins, they get digested by gastric acid and intestinal enzymes, and even if they overcome this barrier, poor permeability remains another problem.[1] Various approaches, including microparticles/nanoparticles, liposomes, gastrointestinal patches, permeation enhancers, and so on, have been tried to address this problem with limited success.[2–4] Therefore, attempts are being made to improve the various strategies to enhance the uptake of peptides to increase bioavailability.

Chitosan is a naturally occurring cationic polymer composed of *N*-acetyl glucosamine and glucosamine, which is widely exploited for various biomedical applications, such as proteins/peptides, gene delivery, and tissue engineering. Chitosan is biocompatible, bioresorbable, and biodegradable and has mucoadhesive properties.[5–7] The hydroxyl groups and its reactive amino group can be easily modified under mild reaction conditions to prepare a modified chitosan with customized properties. In the recent years, chitosan and its numerous derivatives have been investigated extensively for drug-delivery applications. Now, blends of chitosan with various other polymers have also been widely investigated toward for the same applications to improve the matrix properties. Various combinations, such as chitosan and poly(ethylene glycol) (PEG), xanthan, gellan, alginate, and poly lactic glycolic acid (PLGA),[8–10] have been reported. PEG is a biodegradable, hydrophilic polymer that is widely used in the pharmaceutical industry, for applications in transplantation and drug delivery, and also as a component in medical devices.[11] PEG, being nonionic, prevents interaction between other components, and it is a flexible polymer. It was reported that PEG enhances mucoadhesion by providing anchorage for the microparticles in the mucosa.[12] Chitosan–PEG nanoparticles were investigated for nasal insulin-delivery applications. However, the effect of the incorporation of PEG into matrices developed with hydrophobically modified chitosan, such as phthalyl chitosan (PC), has not

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been studied for oral peptide delivery. The phthalate group prevents the dissolution of the chitosan particles and also the release of loaded bioactive molecules at gastric pH. However, PC is slightly hydrophobic due to the bulky aromatic phthalate group, which may adversely affect the swelling and release and also the mucoadhesion of the particles. Aiedeh and Tahab[13] reported the resistance of water intake by chitosan phthalate tablets developed for a diclofenac colon delivery system. The objective of this study was to develop phthalyl chitosan–poly(ethylene oxide) (PCP) semi-interpenetrating polymer network particles for oral insulin delivery. PC is slightly hydrophobic, and poly(ethylene oxide) (PEO) was introduced to improve its loading efficiency and also the *in vitro* release characteristics and mucoadhesion. PEG is known for its biocompatibility and is supposed to improve the biological properties to thereby enhance the stability of the loaded biologically active peptide and also enhance intestinal permeability. The model protein drug used was insulin.

EXPERIMENTAL

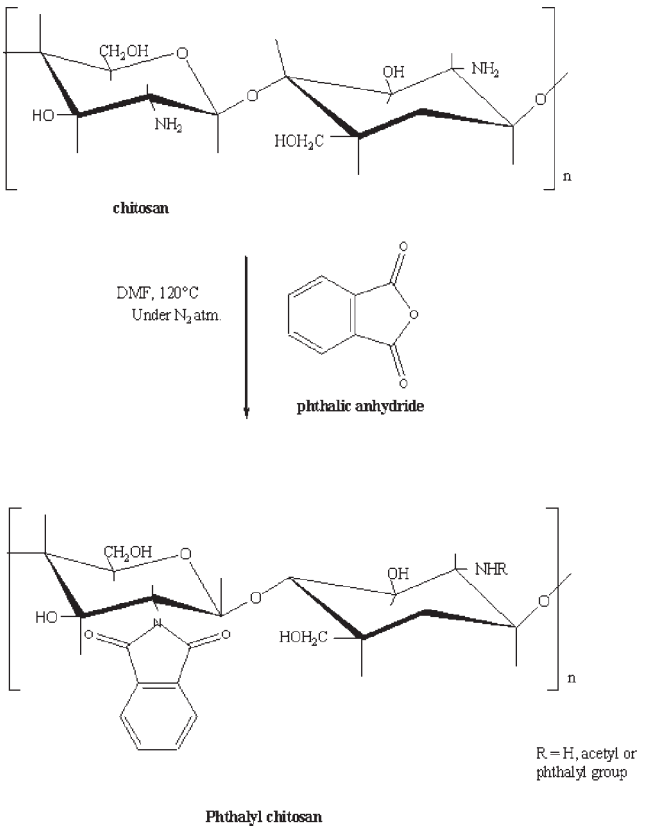
Materials

The materials used included chitosan (85% deacetylated, weight-average molecular weight = 270,000, CIFT, Kochi, India), trinitro benzene sulfonic acid, pig mucin (type III), PEO (weight-average molecular weight = 100,000), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco's Modified Eagles Medium (Sigma-Aldrich Chemical Co., St. Louis, MO), phthalic anhydride, *N,N′*-dimethyl formamide (Merck, Mumbai, India), and fetal bovine serum (Gibco). Human insulin (400 IU/mL) was kindly provided by USV, Ltd. (Mumbai, India). All other reagents used were analytical grade from Merck India.

Methods

PC was prepared by a method previously reported.[14] Chitosan was dispersed in dimethylformamide (DMF) and stirred with a hotplate magnetic stirrer. To this, phthalic anhydride dissolved in DMF was added. The reaction was performed at 120°C for 8 h (Scheme 1). The brown solution obtained was cooled to room temperature, filtered through nylon mesh, and added to ice-cold distilled water. The precipitate obtained was collected by centrifugation at 8000 rpm for 20 min, washed thoroughly with methanol, and dried *in vacuo*.

PC particles were prepared by the dissolution of PC in dichloroacetic acid (1% w/v), to which a 0.6% sodium tripolyphosphate solution was added under high-speed stirring; this continued for 20 min. To prepare the PCP particles, PC was dissolved in



Scheme 1 Preparation of chitosan phthalate.

dichloroacetic acid, and to this, an equal volume of PEO solution (1%) was added under vigorous stirring. Particles were developed by the addition of sodium tripolyphosphate to this solution under high-speed stirring. The resulting suspension was centrifuged at 6000 rpm for 15 min. The pellet obtained was resuspended in distilled water, washed, and centrifuged again for the same period of time at the same speed. The process was repeated three times, and the particles were dried at 2–8°C under refrigerated conditions.

The derivatization was checked with a Fourier transform infrared (FTIR) Impact 410 spectrometer (USA). The chitosan derivatives were used in a powder form, and the spectra were analyzed with the attenuated total reflectance mode.

The ζ potential was measured in folded capillary cells with a nanosizer (Malvern Instruments, Ltd., London, UK). Measurements were performed in buffers of pH 1.2 and 7.4. Each batch was analyzed in triplicate. The particle size was determined on the basis of dynamic light scattering with a particle sizer (CIS-100 particle sizer, Ankersmid, Germany). The surface morphology of the particles was analyzed with scanning electron microscopy (SEM; Hitachi S 2400, Japan).

Dried particles were loaded with insulin (1 mL of insulin/400 mg of particles) by a diffusion filling

method. To the preweighed particles, an insulin solution was added, and this mixture was left for 8 h, after which the excess insulin solution was wiped off. The loaded particles were then dried under refrigerated conditions at 4°C.[15] The loading capacity was determined by the Lowry method.[16] The biological activity of the loaded insulin was analyzed by means of enzyme-linked immuno sorbent assay (ELISA). Particles (100 mg) were dispersed in 10.0 mL of phosphate buffer at pH 7.4 and kept overnight in a refrigerator for complete extraction of the loaded insulin. First, the loaded insulin quantity per 100 mg of particles was estimated in terms of protein content (IU) by the Lowry method. ELISA was done on the same supernatant to estimate the total active insulin units. The protein content and the quantity of the active insulin were both estimated in IU. The kit used was from Mercodia, Sweden (Human), and the absorbance was read with a Finstruments microplate reader (MTX, USA).

The swelling characteristics and insulin release profile studies were done at pH 1.2 and 7.4 [simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), respectively, as per U.S. Pharmacopoeia (USP)] under ambient conditions. The dried test samples were suspended in buffers of the respective pH. At specific time intervals, the samples were removed from the buffer, and after the excess water on the surface was removed, the weight was determined. The degree of hydration of the samples was calculated with the following equation:[17]

$$\text{Degree of hydration} = [(W_s - W_d)/W_d] \times 100\% \quad (1)$$

where W_s is the weight of the swollen particles and W_d is the weight of the dried particles.

For the release studies, the insulin-loaded particles were suspended in respective buffers of pH 1.2 and 7.4. The samples (200 μL) were collected at 1 h intervals for 8 h, and the respective buffers were replaced with the same amount of fresh buffer.

To check the toxicity of the derivative, an MTT assay was done on the L929 cell line.[18] The L929 mouse fibroblast cell line was cultured in Dulbecco's Modified Eagles Medium supplemented with 10% fetal bovine serum and kept at 37°C in a humidified atmosphere of 5% CO_2. Cells were seeded in 24 well plates at a density of 2×10^5 cells/well and incubated for 24 h. The medium was replaced with medium-containing particles (PC and PCP) at a concentration of 1 mg/mL. The cells were incubated for 24 h. The medium-containing particles were removed, MTT was added to the wells at a concentration of 100 μg/well, and the well plates were incubated at 37°C for 4 h. The medium was then removed; 500 μL of DMSO was added, and the cells were again incubated at room temperature for 15 min to dissolve the formazan crystals. We transferred 100 μL of this solution into a 96-well plate reader, and the absorbance was measured with a plate reader (Finstruments microplate reader). The percentage viability was calculated with eq. (2). The medium was used as a control, and 10% phenol was used as a positive control:

$$\text{Viability (\%)} = (\text{Mean absorbance of sample}/\text{Mean absorbance of control}) \times 100 \quad (2)$$

Mucoadhesion studies were conducted with pig mucin type III and with rat intestine. The interaction was studied by the incubation of mucin and microparticles in a neutral medium (pH = 7.4). For the studies with pig mucin, to 1.0 mL (5 mg/mL) of mucin solution, particles were added at a ratio of 1 : 5 (w/w). The incubation was carried out under stirring at 100 rpm and 37°C in an incubator shaker. The dispersions were centrifuged after 2 h at 6000 rpm for 15 min. A-100 μL aliquot was taken, and the protein estimation was done by the Lowry method. According to this procedure, the absorbance of mucin was measured by colorimetry at a wavelength of 750 nm.[19] The amount of the mucin adsorbed by the microparticles was determined as the difference between its initial concentration and the concentration found in the dispersion after incubation and centrifugation. The calculations were made on the basis of mucin standard curves.

Intestinal tissue from male Wistar rats' jejunum approximately 5 cm in length was taken and cut open. The tissue was mounted on a semicylindrical polypropylene support and washed with saline. Twenty-five milligrams each of native chitosan (NC), PC, and PCP particles were spread on the intestinal tissue, and the tissue was kept in a humidity chamber for 5 min. The tissue was then washed with phosphate buffered saline at pH 6.8 for 30 min at an angle of 45°.[20] The dislodged particles were collected and dried, and the weight was noted.

RESULTS AND DISCUSSION

PC was prepared with a well-established procedure from the literature.[14] Anhydrides are highly reactive toward nucleophiles and are able to acylate a number of important functional molecules and other macromolecules. If the anhydride is of a dicarboxylic acid, one of the carboxyl groups will form a covalent bond, and the other will generate a free carboxyl group.

The IR spectra revealed the derivatization of chitosan [Fig. 1(A,B)]. It is known from literature that the characteristic bands of chitosan are 1655, 1560, and 1380 cm^{-1}.[21,22] Dang et al.[22] reported that, for

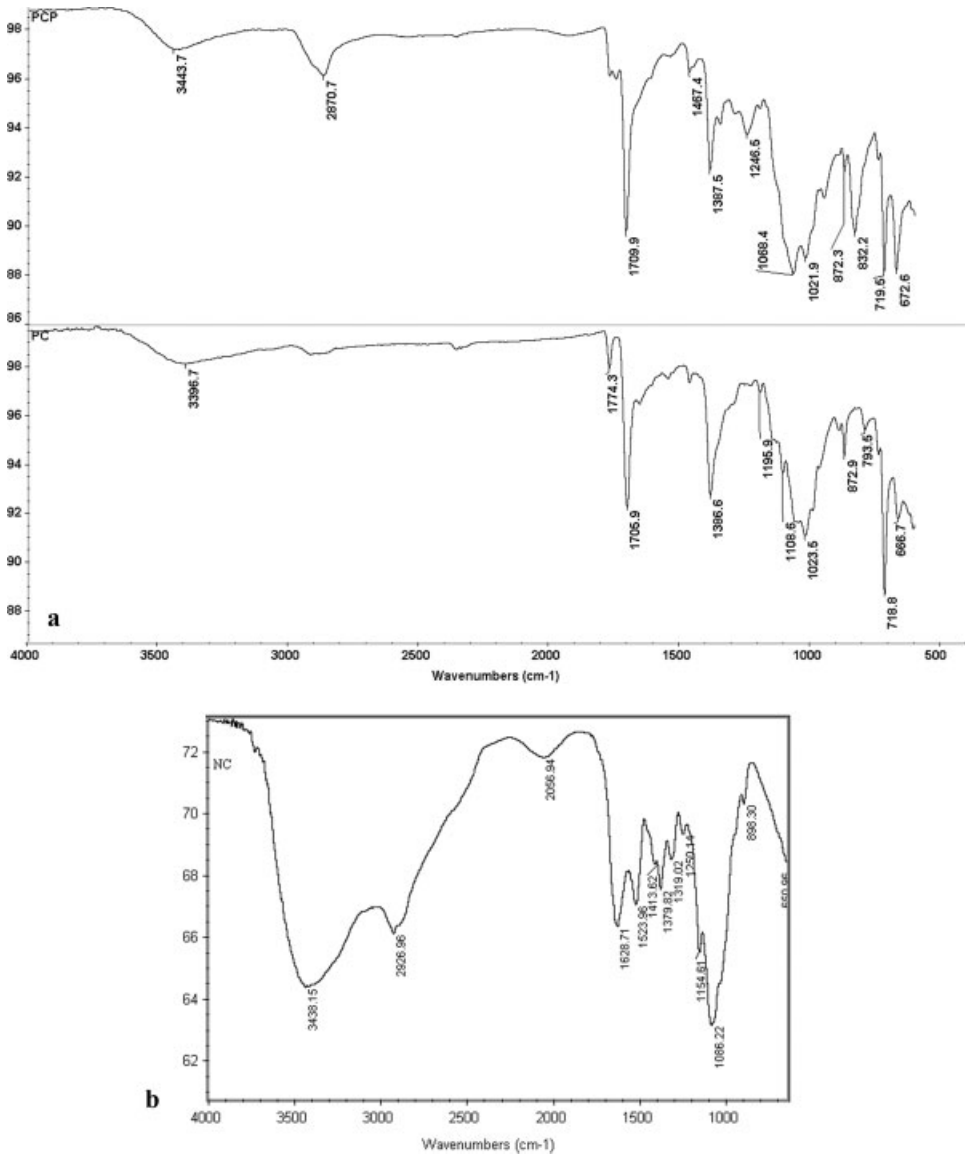


Figure 1 FTIR spectra of (A) PC and PCP and (B) NC.

chitosan, the intensity of the band at 1560 cm^{-1} decreased with deacetylation and became very weak for highly deacetylated specimens because of the increase in the intensity of the band at about 1600 cm^{-1} (>50%). The intensity of the band at 1628 cm^{-1} was higher in NC, as observed in the spectra given. This band disappeared in the derivatized chitosan. A similar observation was reported by Xu et al.;[23] they observed the disappearance of the NH_2-associated band at 1600 cm^{-1} when chitosan was modified. The band at 1706 cm^{-1}, which corresponded to aromatic groups, confirmed the derivatization of the amino groups [Fig. 1(A)]. A similar observation related to carboxyl groups was reported earlier.[13] The band at 2871 cm^{-1} was present in the PCP particles to indicate the presence of PEO.

PC is relatively hydrophobic, and the solubility is higher in solvents such as *m*-cresol, methanol/CaCl, and dichloroacetic acid. The PC solution (1%) was hence prepared in dichloroacetic acid, to which an equal concentration of PEO solution was added, to which sodium tripolyphosphate was added to result in particle formation with the PCP interpolymeric network. An *interpenetrating polymer network* is defined as a combination of two or more polymers to form a network, at least one of which is polymerized and/or crosslinked in the immediate presence of the other.

Particle characterization

The ζ potential values of NC, PC, and PCP were measured at pH values of 1.2 and 7.4. At acidic pH, the particles had no negative charge, and the values were near neutral (Table I). In the *in vivo* conditions, the intestinal pH was near neutral, and hence, the ζ

TABLE I
ζ Potentials of the Microparticles at Different pH Values

	ζ potential (mV)	
Particle	pH 1.2	pH 7.4
NC	—	26.7 ± 5.5
PC	7.1 ± 0.64	−27.8 ± 7.8
PCP	4.1 ± 0.37	−28.6 ± 12.6

potential was determined at pH 7.4. The PC and PCP particles were found to be negatively charged (−27.8 ± 7.8 and −28.6 ± 12.6, respectively) by the ζ potential evaluation at pH 7.4, whereas the ζ potential of NC was positively charged (26.7 ± 5.5). This negative charge may have been due to the presence of carboxyl groups from the introduced phthalate group. The presence of PEO did not make any difference in the net charge of the PC and PCP particles at pH 7.4. Polymers with phthalyl groups, such as hydroxypropyl methylcellulose phthalate and cellulose acetate phthalate, are widely used for the development of enteric coatings to prevent the gastric release of loaded drugs from tablets or capsules. Enteric coatings prevent the release of medication before it reaches the small intestine. Hence, it can be understood that the introduction of a phthalyl group could impart pH sensitivity to chitosan, which is just the opposite that of the NC. However, the phthalyl group, being aromatic and bulkier, has slightly hydrophobic properties, and it has also been reported that PC shows a crystalline nature, which is not exactly suitable for the development of oral peptide-delivery systems, where mucoadhesivity is of great importance. SEM images showed the morphological difference between the PC [Fig. 2 (A,B)] and PCP particles [Fig. 2 (C,D)]. The PCP particles had a porous structure, whereas the PC particles were nonporous. To improve the swelling characteristics, modulate the release pattern, and enhance the mucoadhesivity, PEO was added. As expected, the incorporation of PEO during particle formation improved the loading capacity and the release characteristics. From the Lowry and ELISA data, we found that the quantity of insulin IU was same in both cases, which indicated that the loaded insulin was biologically 100% active. The loadings for the PC and PCP by protein content were 69.4 ± 2.1 and

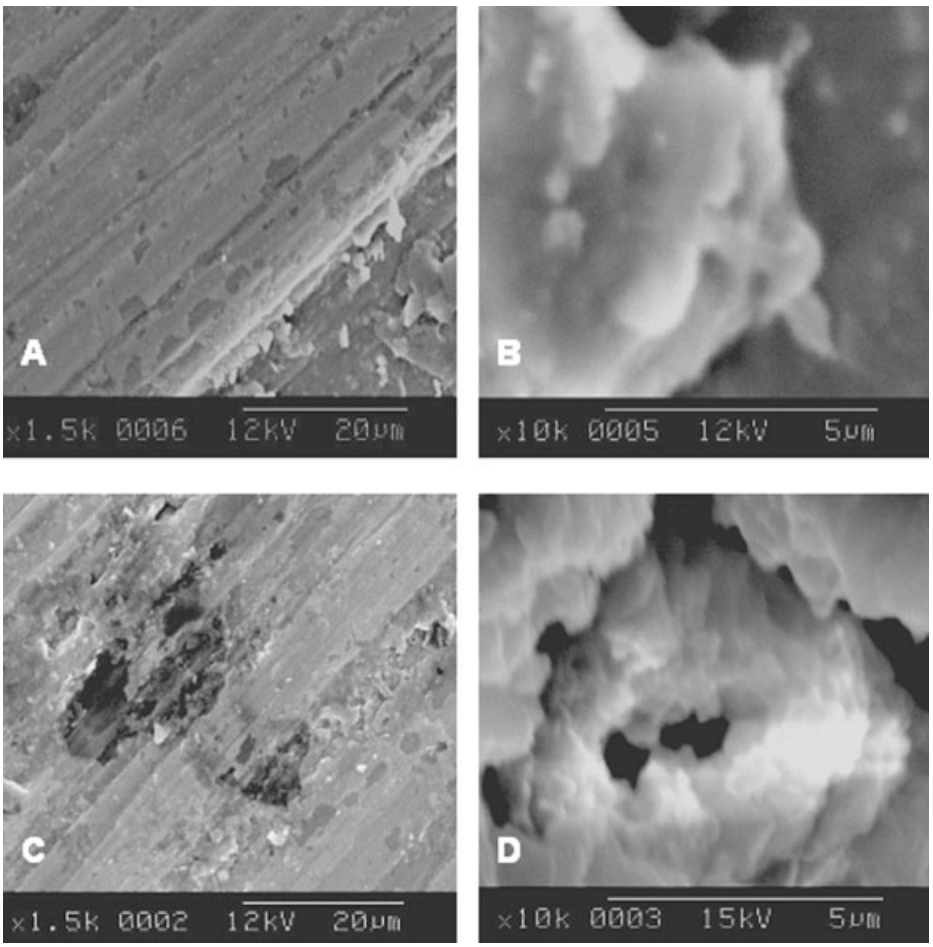


Figure 2 SEM images of (A,B) PC and (C,D) PCP particles at magnifications of 1.5 and 10×.

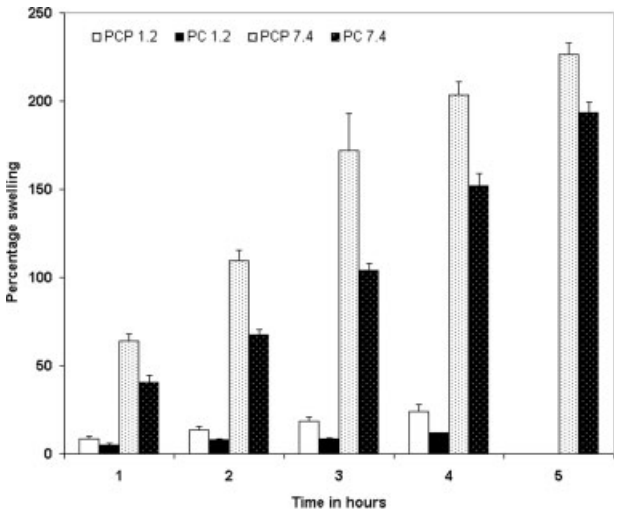


Figure 3 Swelling patterns of PC and PCP particles at pH 1.2 and 7.4 ($n = 3$).

91.4 ± 1.6 IU, respectively, and 67.5 ± 1.5 IU/100 mg and 89.6 ± 3.2 IU/100 mg, respectively, in terms of biological activity. These values showed that the incorporated PEO helped to increase the loading efficiency probably because of higher swelling in the latter case; the difference in the swelling pattern was very obvious from the data (Fig. 3).

The release studies were done in buffers of pH 1.2 and 7.4. The drug-release mechanism from hydrogel matrices is usually through swelling, dissolution, diffusion, or erosion process. Here, the particles were stable even after 24 h. The drug release may be have been done through swelling and diffusion. The release from the PC and PCP microparticles at pH 1.2 was much lower than at pH 7.4 (Fig. 4). After 4 h, the release was only about 17.6 and 22% compared to 56.5 and 66.7% at pH 7.4 for PC and PCP, respectively. It was obvious from the data that the maximum release of insulin was from NC particles at pH 1.2 and that, by virtue of the introduction of phthalyl groups, the insulin release from PC and PCP was significantly minimized. Insulin release from the PCP particles was slightly higher at pH 1.2 and 7.4 compared to that of the PC particles. This might be have been because of the bulkier nature of the particles because of the presence of PEO. Aiedeh et al.[13] reported the lower release of sodium diclofenac from a PC-based colon drug-delivery system and attributed this to the hydrophobic aromatic phthalyl group. Because of this, PC resisted water intake, and correspondingly, the release was also minimized. The data showed that the incorporation of PEO improved the release properties at pH 7.4. About 72.3% of the loaded insulin was released by PC, whereas the release was 87.5% for the PCP particles after 6 h. In both particles, the release was slow and continuous, but for PCP, the percentage release was higher than for PC, which could have been due to the presence of PEO. The carboxylate ions at gastric pH were protonated and, hence, resisted swelling and release, but at intestinal pH, they were negatively charged, and the repulsive forces caused the swelling of the microparticles, which led to the release of loaded insulin. The release pattern was similar in both cases at both pH values.

Cytotoxicity

The *in vitro* cytotoxicity of the particles developed with the derivatives was checked on L929 cell lines with the MTT assay. The cells were exposed to the particles at a concentration of 1 mg/mL. Live cells convert the MTT tetrazolium compound into formazan crystals, and the intensity of the optical density is proportional to the cell viability, which is compared to the values of negative control. The results show (Fig. 5) that, for all of the particles, the cells retained 100% of their metabolic activity as compared with the negative control (medium).

Mucin–microparticle interaction

The microparticles were subjected to mucin interaction studies *in vitro* with pig mucin. The data of this study showed that the incorporation of PEO slightly improved the mucin adsorption properties of the PCP particles. The mucin absorption values for the NC, PC, and PCP particles were 10.28 ± 1.17,

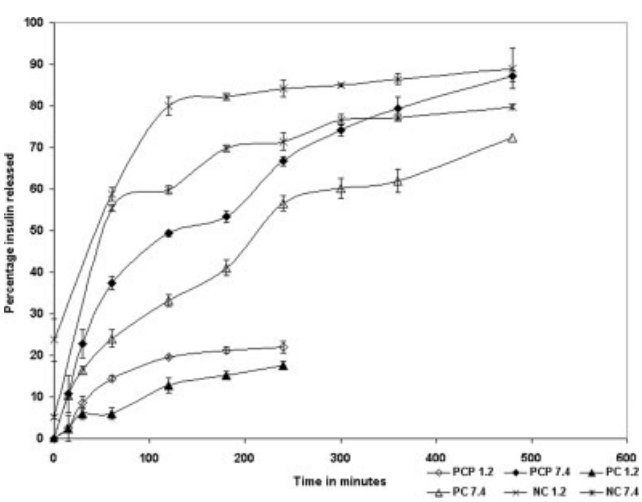


Figure 4 Comparison of the insulin-release kinetics from NC, PC, and PCP particles at both pH 1.2 and 7.4. NC 1.2 and NC 7.4 represent the release profiles of NC particles at pH 1.2 and 7.4, respectively. PC 1.2 and PC 7.4 represent the release profiles of PC particles at pH 1.2 and 7.4, respectively, and PC 1.2 and PC 7.4 represent the release profiles of PC particles at pH 1.2 and 7.4, respectively.

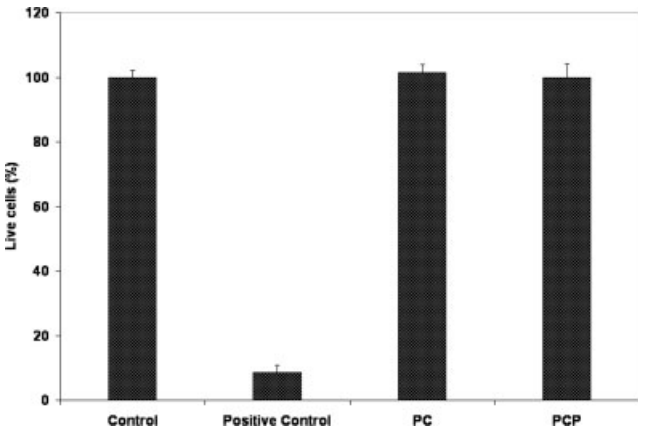


Figure 5 *In vitro* cytotoxicity of PC and PCP particles on L929 cells as measured by the MTT assay. Cell viability is expressed as mean ± standard deviation ($n = 4$).

20.86 ± 3.04, and 11.22 ± 2.1, respectively. Yoncheva et al.[19] developed polyethylene glycolated (PEGylated) nanoparticles based on poly(methyl vinyl ether-*co*-maleic anhydride) and studied their interaction with mucin. They used PEG of two different molecular weights, 1000 and 2000 Da, and these particles also had a negative ζ potential. The NC particles showed comparatively less absorption of mucin; this may have been because of the poor swelling properties of the particles at this pH. The lesser mucin adsorption showed by PCP compared to PC might have been due to steric hindrance from the presence of PEO chains. A similar observation was made and reported earlier by Yoncheva et al.[19] Here, the matrix was of a high swelling degree, which led to the protrusion of PEO chains, which had protein-repellant properties. Hence, this could have been the reason for the low mucin binding to the PCP particles. For PC particles, the mucin adsorption was about 18%, which was higher than for PCP. This might have been due to the exposure of the carboxyl groups of the phthalyl group on swelling, which led to the development of hydrogen bonds or ionic interactions with mucin. Mucoadhesion is proposed to take place by three stages, namely, wetting, interpenetration, and mechanical interlocking between the mucosa and polymer.[24] It is now well known that the polymer–mucin interactions are based on the hydrogen-bond interaction. The poor *in vivo* performance of the oral peptide carriers of known mucoadhesives is attributed to the presence of free mucin in the gastrointestinal lumen.[25] Mucoadhesive polymers bind to the free mucin and get coated and deactivated by the time they reach the mucosal surface. The binding of these polymers to mucin alone is not enough to form a mucoadhesive polymer. The polymer should also be able to reach the mucosal surface in the activated form without binding mucins and be able to bind to the mucosal surface. Recent studies conducted by various group have indicated mucin–mucin repulsion in aqueous meida.[26–28] Therefore, now it is suggested that a successful mucoadhesive should bind to the mucosa rather than to the mucin.[25,29]

In vitro mucoadhesion studies

Mucoadhesion takes place from the interaction between the mucosa and the polymer; it is dependent on the polymer structure and the charge. The *in vitro* evaluation of the mucoadhesive properties of polymeric microspheres is an important criterion for the development of a mucoadhesive drug-delivery system. It is proposed that polymer chains interpenetrate the mucin chains, which leads to the adhesion. The force of hydrogen bonding and the interpenetration into mucin determines the force of attachment. The binding capacities of the chitosan and the derivatized chitosan particles were tested at pH 6.8. Of the 25 mg of PC and PCP particles applied onto the intestinal mucosa, about 57.7 ± 11.3 and 92 ± 1.1% for PC [Fig. 6(A)] and PCP [Fig. 6(B)], respectively, remained attached after 30 min compared to the chitosan particles, which showed around 69.9 ± 1.57% remaining (communicated). Polyacrylates interact with mucus by hydrogen and van der Waals bonds, which are created between the

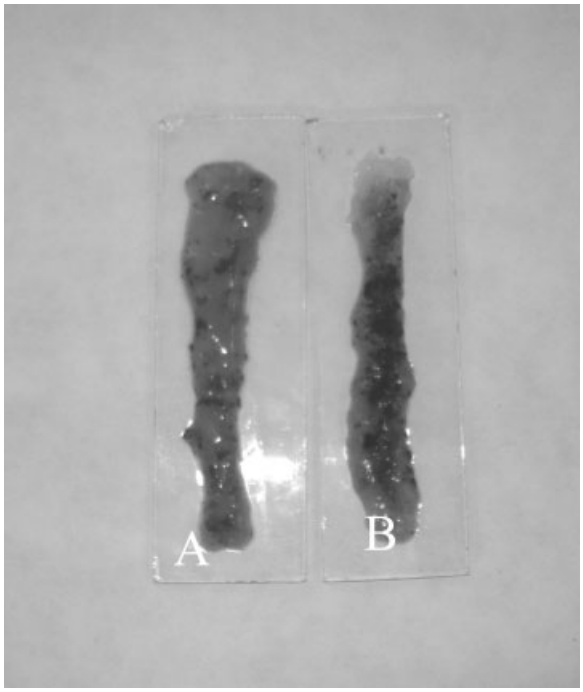


Figure 6 Photograph depicting the mucoadhesiveness: a comparison between the (A) PC and (B) PCP particles. The figure depicts the adhesiveness of the particles after 30 min of washing with phosphate buffer at pH 6.8. The particles adhered were higher in number in PCP.

carboxylic groups of the polyacrylates and the sialic acid residues of mucin glycoprotein. Chickering and Mathiowitz[30] reported that polymers with carboxylic acid groups might produce strong bioadhesive bonds. The strength of the interaction is dependent on the concentration of polar groups, such as hydroxyl and carboxyl groups, and with greater concentration, the mucoadhesive bonds are more intense. It was reported that, from mucoadhesive studies with polyacrylate-based polymethacrylic acid-chitosan-polyether (polyethylene glycol-polypropylene glycol copolymer) (PMCP) nanoparticles, about 84% of particles were retained on the mucosal surface. The noncovalent interactions of carboxyl groups with the hydroxyl groups of mucosa were proposed to be the reason for the strong mucoadhesiveness.[15] The amino groups of chitosan are crosslinked with anionic groups, which leads to the masking of the positive charges of chitosan and results in the reduction of mucoadhesive properties.[31] The NC particles showed a low mucin-binding capacity, and correspondingly, the mucoadhesion was slightly better than that of the PC particles; PCP showed the strongest mucoadhesion. It was reported that microparticles containing free PEO show increased mucoadhesive capacity. Ascentis et al.[32] demonstrated that the mucoadhesion in PEO-loaded poly(2-hydroxyethyl methacrylate) (PHEMA) particles occurred by the interpenetration of the free PEO chains. The authors observed that the PEO-loaded PHEMA particles, although crosslinked, exhibited very high values of mucoadhesive capacity. They interpreted that, even when PEO was loaded in the PHEMA particles, the linear PEO chains could penetrate across the polymer–mucus interface because of its concentration gradient. The PC and PCP particles in our study showed different degrees of swelling, with PCP having a degree of hydration of about 109.3 ± 6.1 at 2 h compared to that of 67.5 ± 3.2 for PC. This might have been due to the water uptake and swelling of the particles, which allowed the loaded PEO to penetrate into the mucosa and ultimately increase the mucoadhesion. The PC particles were more of a crystalline nature, and the bulkier aromatic groups may not have been able to penetrate easily into the mucosa or establish proper anchoring on the mucosal surface, which led to decreased bioadhesion. Also, as shown from the mucin adsorption studies, the PC particles tended to bind more mucin than PCP, which has been reported to affect the mucosal binding.

CONCLUSIONS

Phthalyl and PCP particles were developed as oral insulin-delivery matrices. The particles showed improved release properties compared to chitosan particles. Insulin release in acidic media was minimized because of the presence of the phthalate group. Both the PC and PCP particles were found to be nontoxic by the cytotoxicity studies. PEO was introduced during particle preparation with the aim of enhancing the loading and improving the release characteristics for insulin and also the mucoadhesive properties. In both cases, the release was slow and continuous, but for PCP, the percentage release was higher than for PC, which could have been because of the presence of PEO. The PCP particles showed higher swelling and enhanced mucoadhesivity compared to the PC particles. From these studies, it seems that PCP may be a good candidate for the oral delivery of insulin and other proteins.

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