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Polymerbiocomposite chitosan nanoparticles for sustained aceclofenac drug delivery

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ABSTRACT

Nanoparticles of ~10 nm in diameter made with chitosan or lactic acid-graftedchitosan were developed for high drug loading and prolonged drug release. A drug encapsulation efficiency of 94.4% and a release rate of 28% from chitosan nanoparticles after 24 hours were demonstrated with bovine serum protein. A drug encapsulation efficiency of 97% and a release rate of 38% from chitosan nanoparticles after 24 hours were demonstrated with aceclofenac. To further increase drug encapsulation, prolongdrug release, and increase chitosan solubility in solution of neutral pH, chitosan was modifiedwith lactic acid by grafting D,L-lactic acid onto amino groups in chitosan without using a catalyst. The lactic acid-grafted chitosan nanoparticles demonstrated a drug encapsulation efficiency of96% and a protein release rate of 15% after 24 hours. And in case of diclofenac, drug encapsulation efficiency of 98% and a aceclofenac release rate of 25% after 24 hours. Unlike chitosan, whichis generally soluble only in acid solution, the chitosan modified with lactic acid can be preparedfrom solutions of neutral pH, offering an additional advantage of allowing proteins or drugs tobe uniformly incorporated in the matrix structure with minimal or no denaturization.

ntroduction

In recent years, significant efforts have been devoted to use the potentials of nanotechnology in drug delivery since it offers a suitable means of site-specific and/or time controlled delivery of small or large molecular weight drugs and other bioactive agents (Soppimath KS, *et al.*, 2001). Pharmaceutical nanotechnology focuses on formulating therapeutically active agents in biocompatible nano forms such as nanoparticles, nanocapsules, micellar systems, and conjugates. These systems offer many advantages in drug delivery, mainly focusing on improved safety and efficacy of the drugs, e.g. providing targeted delivery of drugs, improving bioavailability, extending drug or gene effect in target tissue, and improving the stability of therapeutic agents against chemical/enzymatic degradation (Calvo P, *et al.*, 1997).

Chitosan is a natural polymer, non-toxic, edible, biodegradable, derived by deacetylation of chitin, the second most abundant biopolymer in nature after cellulose (Dodane V, and Vilivalam VD. 1998). Chitosan has been used in edible coatings or films to extend the shelf life of foodstuffs, e.g. fruit, meat, and fish and seafood. However, its high sensitivity to moisture limits its applications for packaging. Blending chitosan with other biodegradable polymers, for example, poly (3-hydroxybutyric acid) (PHB), poly (ε-caprolactone) (PCL) and poly lactic acid (PLA) has been used to modify its water sensitivity properties (Janes P.C, *et al.*, 2001). The strategy to overcome this drawback is the blending of chitosan with moisture-resistant polymers, while maintaining the overall biodegradability of the products. Suyatma*et al*. (2004), reported on biodegradable film blends of chitosan with PDLLA by solution mixing and film casting.

Chitosan was selected for nanoparticles because of its recognized mucoadhesivity and ability to enhance the penetration of large molecules across mucosal surface (Xu .Y, *et al.*, 2003). Chitosan nanoparticles are obtained by the process of ionotropic gelation based on the intraction between the negative groups of sodium diclofenac and the positively charged amino groups of chitosan (Berthold A, *et al*., 1996). This process has been used to prepare chitosan nanoparticles for the delivery of peptides and proteins including bovine serum albumin insulin, cyclosporine and many other plant extracts (Amir Dustgania, *et al.*, 2008).

In this study, chitosan-based nanoparticles with a high degree of size uniformity were prepared by grafting D,L-lactic acid on chitosan to serve as a drug carrier for prolonged drug release.

The lactic acid-grafted chitosan (LA-g-chitosan) was prepared by dehydrating the solvent cast thin film of chitosan containing lactic acids. The LA-g-chitosan nanoparticles were fabricated using co-precipitation process by LA-g-chitosan in ammonium hydroxide to form coacervate drops. The structure of nanoparticles was investigated by transmission electron microscopy (TEM). Encapsulation of bovine serum albumin (BSA) and aceclofenac were done with chitosan nanoparticles for analysing the drug uptake and drug releasing ability of chitosan.

Experimental procedure

Materials

Chitosan from crab shells with 75% of deacetylation (weight average molar mass MW 190 KDa and EC 222- 311-2) was purchased from Sigma- Aldrich Co. USA. Bovine Serum Albumin (MW 68kDa) and D, L- Lactic acid (90% aqueous

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solution) were purchased from Genei co. India. aceclofenac, was purchased from Cipla Pharmaceutical Co., India.

Preparation of lactic acid grafted (LA-g-Chitosan) chitosan copolymer

0.2g of chitosan powder was mixed with an aqueous solution of 10ml poly-D,L-lactic acid and the mixture was stirred overnight using a magnetic stirrer to prepare a final solution at a chitosan concentration of 2wt%. The solution was placed in petridishes and maintained at 70^{o}C for 5 hours to form a thin film. The formed film was heated at 80-90^{o}C for 5 hours.

Characterisation of LA-g-chitosan copolymer

FTIR spectroscopy was used to characterize bonding characteristics of LA-g-chitosan copolymer. Dried 5mg sample was mixed with 300mg dry KBr and pressed into a pellet using a macro KBr dye kit. The solid pellet was placed in a magnetic holder and the system was purged with air before testing. FTIR spectra of 200 scans at 4cm^{-1} resolution were acquired using a Nicolet 5DX spectrometer equipped with a deuteratedtriglycinesulphate (DTGS) detector and a solid transmission sample compartment. Spectrum analysis and display were performed using standard Nicolet and Microcal Origin software.

Nanoparticle preparation from chitosan and LA-g-chitosan

Chitosan nanoparticles were prepared by separately dissolving chitosan powder and LA-g-chitosan film in 0.2M acetic acid to produce 2wt% solution. All the two solutionswere added individually withammonium hydroxide solution (pH 8.5-9.0). A constant solution flow rate of 0.1ml/minute was maintained using a syringe.

As the acetic chitosan solution was introduced into the basic solution, the opalascent suspension was formed under vigorous magnetic stirring at room temperature for all the two samples. The particles were separated from the solution by centrifugation (10,000rpm for 15 minutes). After separation, the particles were washed with deionized water until a pH of 7.4 was reached.

Morphological characterization of nanoparticles

The morphology and size distribution of nanoparticles were examined by TEM (CM 100 TEM) at an accelerating voltage of 40 kV. Samples were prepared by depositing a drop of chitosan nanoparticle suspension on a copper grid fitted with a carbon support film and dried under vacuum.

BSA and aceclofenac absorption on Nanoparticles

100mg of LA-g-chitosan and chitosan nanoparticles were dispersed separately in 3ml of phosphate-buffered saline (PBS) with a pH of 7.4, under sonication (5 cycles, 30amplitudes for 2 min). 5mg/ml of BSA and aceclofenac was added to all the two sonicated solutions individually.

The nanoparticle suspensions were left for 24 hours in a rotating agitator for nanoparticle absorption of loaded samples. After absorption, the nanoparticles were separated by centrifugation (20,000rpm for 15 min) and the amount of free protein and aceclofenac the supernatant of both the sonicated solutions were measured using Coomasie blue protein assay and aceclofenac assay by UV spectroscopy at 590nm and 276nm respectively.

Encapsulation efficiency (AE) was calculated as,

$$AE = \frac{(T-F) \times 100}{T} (\%)$$

Where, T → Total amount of sample added in PBS

F → Amount of non-absorbedsample left in supernatant after removing nanoparticlesfrom the solution.

Coomasieblue protein assay

4mg of BSA was dissolved in 2ml of PBS (pH-7.4) as stock solution. Using stock, the standards were prepared to have different concentrations of BSA (0-2000 µg/ml).0.1ml of each standard sample was added with 5ml of Coomasie reagent mixed and incubated for 10 minutes at room temperature. The absorbance was measured by UV- visible spectrophotometer at 590nm.

aceclofenac assay

aceclofenac stock was prepared by dissolving 5mg of aceclofenac in 100ml of PBS and the solution was filtered off. Using stock, the standards were prepared to have different concentrations of aceclofenac. The absorbance of each standard was measured by UV-Visible spectrophotometer at 276nm.

***In vitro* release of BSA and aceclofenac Nanoparticles**

BSA and aceclofenac loaded nanoparticles were placed in 3ml of PBS (pH-7.4) and incubated at 37˚C for 24 hours. At specified time intervals, the supernatant was removed, and the medium in the test tube was replenished with fresh PBS. The amount of BSA and aceclofenac released from the Nanoparticles was measured at 590nm and 276nm respectively.

Results and Discussion

Characterization of LA-g-chitosan polymer

LA-g-chitosan copolymer films were obtained by using lactic acid as copolymer. Copolymer film obtained was transparent and readily soluble in solutions with pH up to 7.5 (Fig.1). During the dehydrating process, the formation of amide and polycondensation of lactic acid took place at the same time (Janes PC,*et al.,* 2001). The unreacted lactic acid and oligomeric lactic acid in the reaction mixture were washed out as impurities using excess chloroform and methanol.

Fig:1 Preparation of D,L-Lactic acid grafted chitosan film

Fig.1(a) **Fig.1(b)**

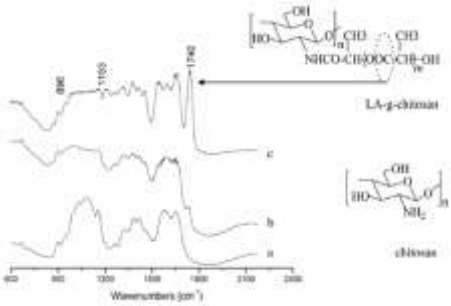
The co-polymer transparent film which was prepared by dehydrating (Fig.1a) and washing with chlooloroform and methanol (Fig.1b).

Figure 2, shows FTIR spectra of chitosan and LA-gchitosan with two different ratios of lactic acid to chitosan. The peaks at 1655 and 1325 cm^{-1} in the IR spectrum of chitosan correspond to amide I and amide III, respectively (Fig.2a). The peak at 1585 cm^{-1} is the free amino band of chitosan. The two peaks at 902 and 1157 cm^{-1} are the result of the saccharide structure of chitosan. The peak at 1377 cm^{-1} is the characteristic band of CH_3 symmetrical deformation mode. The LA-g-chitosan spectrum (Fig.2b) has a broad band around 1591cm^{-1}, owing to the overlapping of the peaks from the free amino band of chitosan and the amide that couples chitosan and lactic acid oligomers. The peak at 1736 cm^{-1} is attributed to carbonyl of ester or carboxylic groups on the lactic acid side chains. When LA-g-chitosan was prepared with a high content of lactic acid (Fig.2c), the broad band around 1591 cm^{-1} was absent and multiple peaks appeared corresponding to the *N*-acetylated and free amino groups of chitosan. The increased intensity of the amide I peak (1655 cm^{-1}) indicated an increase in amidation

resulting from the reaction of chitosan with increased amount of lactic acid.

The peak of the amino groups shifted slightly from 1591 to 1598 cm^{-1}, while the intensity of the corresponding peak of the carbonyl group at 1740 cm^{-1} increased. Compared with the reported IR spectrum of lactic acid and their oligomers (Kister*et al*., 1998), the well-resolved peaks at 1452 and 1315 cm^{-1} were attributed to -CH_3 and -CH groups in LA-g-chitosan (Narayan Bhattarai*et al*., 2006).

Fig: 2. Characterisation of Nanoparticles using FTIR



Infrared spectra of (a) chitosan, (b) LA-g-chitosan with BSA and (c) LA-g-chitosan with sodium diclofenac.

Preparation of nanoparticles

Chitosan-based nanoparticles with a high degree of size uniformity were prepared by grafting D,L-lactic acid on chitosan to serve as a drug carrier for prolonged drug release.

The lactic acid-grafted chitosan was prepared by dehydrating the solvent cast thin film of chitosan containing lactic acids. The LA-g-chitosan nanoparticles were fabricated via a co-precipitation process by LA-g-chitosan in ammonium hydroxide to form coacervate drops (Fig.3).

Fig:3 Preparation of Nanoparticles

Fig.3(a) Fig.3(b) Fig.3(c)

The dissolved co-polymer film (Fig.2a) and chitosan powder (Fig.2b) was used to prepare nanoparticles by adding with ammonium hydroxide solution and centrifuge settled (Fig.2c).

Characterization of nanoparticles

Figure 4, shows an exemplary TEM image of LA-g-chitosan nanoparticles at two magnifications.

TEM images of pure chitosan nanoparticles were not provided since they appeared similar to the LA-g-chitosan nanoparticles.

From TEM images, both chitosan and LA-g-chitosan nanoparticles were spherical and have an average diameter of ~10 nm and a fairly uniform size distribution.

Advantages of using small-size particles as a carrier system include high cellular uptake, good suspensibility, and easy penetration into arterial walls (Labhasetwar*etal.*, 1997). Furthermore, the particles with smaller sizes have larger surface area to volume ratios and thus may have a high drug-loading capacity and a slow drug-diffusion rate.

Fig:4. Morphology of Nanoparticles in TEM

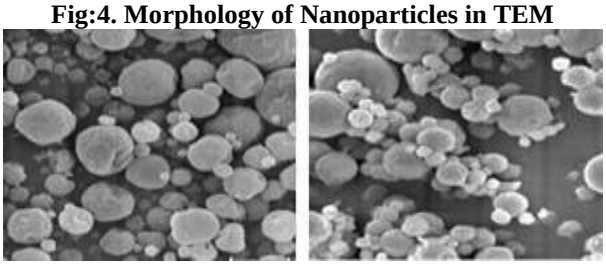


Fig.4(a) Fig.4(b)

The electron microscopic images of LA-g-chitosan nanoparticles (Fig.3a) and chitosan nanoparticles (Fig.3b)

BSA and aceclofenac encapsulation (AE) and interaction with chitosan

Drug AE of chitosan and LA-g-chitosan nanoparticles in BSA solutions was evaluated following the procedure outlined by Narayan Bhattarai*et al.*,(2006) (Fig.5). The AEs of both chitosan and LA-g-chitosan nanoparticles in BSA solution with a BSA concentration of 5 mg/mL were measured to be 94.4% and 95.6%, respectively. Similarly, the AEs of both chitosan and LA-g-chitosan nanoparticles in aceclofenac solution with a aceclofenac concentration of 5 mg/mL were measured to be 96% and 98%, respectively. The high AEs of chitosan and LA-g chitosan may be attributed to the small size of particles that have a high surface to volume ratio and high electrostatic interaction between the negatively charged moieties of BSA and aceclofenac with the positively charged amine groups on chitosan. The fact that LA-g-chitosan nanoparticles had a higher AE than chitosan nanoparticles may be attributed to additional hydrophobic interactions of the LA-g-chitosan particles with BSA and aceclofenac. In the present work, no linker molecules were used to bind the drugs with the polymer and thus only the adsorption owing to the electrostatic attractions between BSA proteins and nanoparticles and the direct entrapment of protein into the polymer matrix would contribute to the drug loading. This helps retain the drug integrity and biofunctionality(Borchard G, *et al.*, 1996).

Fig:5 Encapsulation of Nanoparticles with BSA and aceclofenac

Fig.5(a) Fig.5(b)

LA-g-chitosan (Fig.5a) and chitosan nanoparticles (Fig.5b) were dispersed separately in 3ml of phosphate-buffered saline (PBS) with a pH of 7.4, under sonication (5 cycles, 30 amplitudes for 2 min)

Drug Delivery analysis of BSA and aceclofenac

The percent cumulative release rate of BSA from chitosan and LA-g-chitosan nanoparticles loaded with BSA over various time intervals. For both types of nanoparticles, the protein release rates increased over every time interval. The release of BSA from nanoparticles is mainly driven by the protein concentration gradient. The encapsulation of high concentration produced a greater concentration gradient between the polymer and release medium leading to a higher diffusion rate (Miyazaki *et al*., 1988). All the drug-release profiles exhibit an initial burst release, presumably from the particle surface, followed by a

sustained release driven by diffusion of the protein through the polymer wall and polymer erosion. The release profiles of chitosan and LA-g-chitosan particles are similar, but the BSA release rate from chitosan nanoparticles is significantly higher than from LA-g-chitosan nanoparticles. For example, the burst releases from chitosan and LA-g-chitosan loaded with BSA at a concentration 5mg/mL were about 15% and 10%, respectively. The BSA releases from chitosan and LA-g-chitosan after 24 hours at BSA concentration of 5 mg/mL were 28% and 15%, respectively (Table-1 & Figure-6).The sustained BSA releases from both chitosan and LA-g-chitosan nanoparticles might be attributed to strong intermolecular interactions, including hydrogen bonding and dipole–dipole interactions between chitosan and BSA molecules.

Fig: 6. BSA release profile

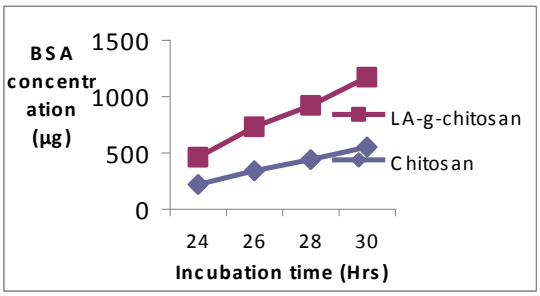
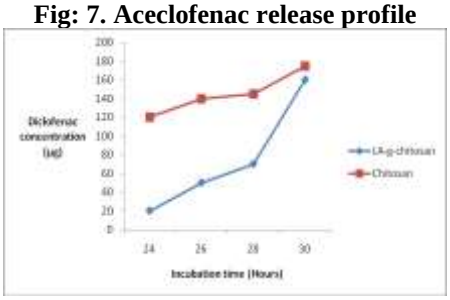


Plate-3 shows the percent cumulative release rate of aceclofenac from chitosan and LA-g-chitosan nanoparticles loaded with aceclofenac over various time intervals. For both types of nanoparticles, the protein release rates increased over every time interval. The release of aceclofenac from nanoparticles is mainly driven by the protein concentration gradient. The encapsulation of high concentration produced a greater concentration gradient between the polymer and release medium leading to a higher diffusion rate (Miyazaki *et al*., 1988). All the drug-release profiles exhibit an initial burst release, presumably from the particle surface, followed by a sustained release driven by diffusion of the protein through the polymer wall and polymer erosion. The release profiles of chitosan and LA-g-chitosan particles are similar, but the aceclofenac release rate from chitosan nanoparticles is significantly higher than from LA-g-chitosan nanoparticles.

For example, the burst releases from chitosan and LA-g-chitosan loaded with aceclofenac at a concentration, 5mg/mL were about 38% and 15%, respectively. The aceclofenac releases from chitosan and LA-g-chitosan after 48 hours at aceclofenac concentration of 5 mg/mL were 20% and 15%, respectively (Table-2 & Figure-7).The sustained aceclofenac releases from both chitosan and LA-g-chitosan nanoparticles might be attributed to strong intermolecular interactions, including hydrogen bonding and dipole–dipole interactions between chitosan and aceclofenac molecules.

Previous studies (Miyazaki *et al.*, 1988) suggested that the mechanism of association of proteins with chitosan is, at least, partially mediated by the ionic interaction between chitosan and protein macromolecules. It is known that lactic acid oligomers are hydrophobic, and introduction of this hydrophobic moiety to chitosan could substantially alter the physicochemical properties of the chitosan nanoparticles for drug absorption (Behzad Sharif MakhmalZadeh, *et al.*, 2010). Thus, the slower drug release from LA-g-chitosan than chitosan might be attributed to additional intermolecular forces, including hydrophobic interactions and hydrogen bonding.

Fig: 7. Aceclofenac release profile



Conclusion

Spherical and uniformly dispersed chitosan and lactic acid-modified chitosan (LA-g-chitosan) nanoparticles with a mean diameter of ~10 nm were prepared. Albumin encapsulation efficiency, as high as 94.4% and 95.6% was attained for chitosan and LA-g-chitosan nanoparticles, respectively. Similarly, the encapsulation efficiency of aceclofenac was attained as high as 96% and 98% for chitosan and LA-g-chitosan nanoparticles, respectively. The BSA and aceclofenac release profiles of both chitosan and LA-g-chitosan nanoparticles exhibit an initial burst release, followed by a sustained quasi-linear release. The chitosan nanoparticles have a protein release rate of 28% over 48hrs and predicated complete protein release up to 8-10days, while LA-g-chitosan nanoparticles had a protein release rate of 15% at the same protein concentration over 48hrs and predicated complete release up to 15-20days. The chitosan nanoparticles releases aceclofenac at a rate of 20% over 48hrs and predicated complete protein release up to 12-15 days, while LA-g-chitosan nanoparticles had a aceclofenac release rate of 15% at the same drug concentration over 48hrs and predicated complete release up to 15-20 days. The resulting release pattern of the protein and drug from LA-g-chitosan nanoparticles showed similar release profile percentage. Hence LA-g-chitosan nanoparticles have different encapsulation potential for the protein and drug but same release profile for them. By incorporating the lactyl segment into the chitosan backbone, the resulting nanoparticle reduces the burst release, but the release patternwas similar to that of the pure chitosan nanoparticles. Since most proteins and cell membranes are negatively charged, these nanoparticles are also expected to be potential vehicles to associate more easily with other proteins and drugs and subsequently internalized by the target cells than negatively charged nanoparticles.

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Table: 1. *In vitro* release of BSA

Time interval of drug release analysis (Hrs)	BSA released from film nanoparticles (µg)	BSA released from chitosan nanoparticles (µg)
24	220	240
26	340	390
28	440	480
30	550	620

Table: 2. *In vitro* release of Aceclofenac

Time interval of drug release analysis (Hrs)	aceclofenac released from film nanoparticles (µg)	Diclofenac released from chitosan nanoparticles (µg)
24	20	I20
26	50	140
28	70	145
30	160	175