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Acyl modified chitosan derivatives for oral delivery of insulin and curcumin

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Abstract In the present investigation, bioadhesive property of chitosan (CS) was enhanced by the N-acylation with hexanoyl, lauroyl and oleoyl chlorides. The chemical structure of the modified polymer was characterized by FTIR and zeta potential measurements. The swelling ability was evaluated at alkaline pH. Mucin interactions and mucoadhesion experiments were performed under in vitro experimental conditions. Cytotoxicity experiments were employed to confirm the applicability of these particles as drug carriers. Finally in vitro evaluation of hydrophobic and hydrophilic drug release profile at acidic and alkaline pH was also conducted. A strong interaction between CS acyl derivatives and mucin was detected, which was further confirmed by an in situ mucoadhesion experiments with excised intestinal tissue. CS modified with oleoyl chloride showed better mucoadhesion property, as compared to the one modified with lower fatty acid groups. CS derivatives were found non-toxic on L-929 cell lines and provided sustained release of hydrophobic drugs under in vitro experimental conditions. From these studies it seems that hydrophobically modified CS is an interesting system for drug delivery applications.

1 Introduction

Polymeric nanoparticles are promising candidates for advanced drug delivery applications. Chitosan (CS) is a unique polymer which is of significant interest in biomedical applications, due to their favourable physicochemical and biological properties [1]. CS is a linear copolymer consisting of β-(1,4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glycopyranose.

CS has been extensively investigated for its potential in the development of drug delivery systems, because of its favourable characteristics like biodegradability [2, 3], biocompatibility [4, 5] and non-toxicity [5, 6]. These properties make CS a good candidate for use in advanced drug delivery applications [7]. Moreover CS chains have good adhesive properties on mucosal surfaces, which can be exploited for the development of bioadhesive delivery devices. CS have hydroxyl and amino functional groups, which allows the formation of hydrogen bonding with the mucus glycoprotein, whereas the linear molecule expresses sufficient chain flexibility to allow the inter-diffusion of polymer chains across the intestinal mucosa [8]. However the major drawback of the CS is its poor solubility at physiological pH. CS is soluble only at aqueous acidic solutions having pH < 6.5 and chemical modifications is usually employed to enhance its solubility. The presence of reactive amino groups is highly beneficial for chemical modification approach.

Long chain acyl derivatives of CS are interesting hydrophobic modification for developing nanoparticles. N-acylation of CS with various fatty acid chlorides increases the hydrophobic character of the resulting polymers. The solubility of the derivative varies with the acyl chain length and also on the degree of substitution. CS derivatives with short chain length (up to C8) with low to moderate degree of substitution exhibit solubility in water, however one with higher degree of substitution display very little or no solubility in water. Because of increase of hydrophobic character, acyl CS derivative with longer chain length is water insoluble, regardless of the degree of substitution.

In this study, hydrophilic and hydrophobic drug was encapsulated onto the hydrophically modified CS

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nanoparticles. Insulin was chosen as a hydrophilic drug and curcumin was selected as a hydrophobic compound. Curcumin a multifunctional agent is an orange-yellow component of turmeric (Curcuma longa). Curcumin have shown to exhibit numerous activities which include anti-inflammatory [9, 10], antimicrobial, antiviral, anticancer [11], antioxidant, chemosensitizer [12], radiosensitive and wound healing. However poor solubility in aqueous medium makes the formulation of curcumin difficult and use of hydrophobic systems have been usually employed in this aspect.

Acylation with fatty acid having carbon atoms C_6–C_{16} increases the hydrophobic character of CS. The aim of the work was to modify the CS molecules by attaching long chain alkyl groups to amino groups. Application of acyl-CS nanosystems towards oral insulin delivery was evaluated. Effect of hydrophobic modification was evaluated by grafting three different fatty acid chlorides on the CS backbone and their effect on particle size, swelling, insulin, curcumin loading/release and mucoadhesion behaviour was evaluated. Cytotoxicity of the modified material was evaluated on L-929 cell lines.

2 Materials and methods

2.1 Materials

CS with molecular weight of 270 kDa and 88% deacylated was purchased from Indian Sea Food (Cochin, India) and used after purification. Hexanoyl chloride ($CH_3{\cdot}(CH_2)_4COCl$) and lauroyl chloride ($CH_3{\cdot}(CH_2)_{10}COCl$) were purchased from Fluka and oleoyl chloride ($CH_3(CH_2)_7{=}CH(CH_2)_7COCl$) was purchased from Merck. Curcumin was obtained from Sigma.

2.2 Acyl modification of chitosan

CS solution (1%) was prepared in 0.1 M acetic acid solution with overnight stirring to ensure complete solubility. pH was adjusted to neutral by slow addition of 0.5 M NaOH with vigorous stirring. Acid chlorides (hexanoyl/lauroyl/oleoyl chlorides) (200 mM) was mixed with the neutralised CS solution and reaction was allowed to continue overnight. Thereafter reaction medium was neutralised with the addition of dil. NaOH and CS derivative so obtained were precipitated in acetone. The crude products like hexanoyl CS (HC), lauroyl CS (LC) and oleoyl CS (OC) were filtered, washed thrice with hot methanol to remove unreacted acid chlorides. Finally the product was dried under vacuum and stored at room temperature.

HC, LC and OC micro particles were prepared by ionic cross linking with tripolyphosphate. HC, LC and OC solutions of 0.2% (w/v) were prepared in 1% (v/v) acetic acid solution. Tripolyphosphate solution (0.2%) was added drop wise to the above solutions with magnetic stirring. The resulting particles were separated by centrifugation and finally freeze dried.

2.3 Characterization

2.3.1 FTIR

FTIR spectroscopy was utilized to characterise CS and acyl-CS derivatives. FTIR was obtained with Nicolet Impact 410 FTIR spectrometer. Native CS and acylated CS were mixed with KBr and pellets were made to record the IR spectra (600–4,000 cm^{-1}).

2.3.2 Particle size analysis

The hydrodynamic mean diameter of the particles was determined by Dynamic Light Scattering using Zetasizer (Malvern, UK). All dynamic light scattering measurements were performed at 25°C with an angle detection of 173°. Six subsequent measurements were made for each sample.

2.3.3 Zeta potential

The zeta potential of the derivatives was determined in different pH conditions (1.2, 6.8 and 7.4) by using Malvern Zeta sizer. Measurement was performed at 25.0 ± 0.1°C and six measurements were taken for each sample.

2.3.4 TNBS assay

The degree of substitution was determined using trinitrobenzenesulphonic acid (TNBS) assay [13, 14].

2.3.5 Swelling studies

Swelling studies of CS and CS derivatives was evaluated in phosphate buffer solution at pH 7.4. Pre-weighed particles were placed in phosphate buffer solution and at regular interval of time excess buffer were removed and the weight of the swollen particles was noted.

Percentage swelling was calculated using the formula

$$\%\ \text{of swelling} = \frac{\text{weight of the swollen particle} - \text{weight of dry particle}}{\text{weight of dry particle}} \times 100.$$

2.3.6 Mucoadhesion studies

2.3.6.1 Mucin binding study Mucin binding experiments was performed to assess the amount of mucin adsorbed on the micro particles [15]. Particles (5 mg) were dispersed in the 1 ml mucin solution (5 mg/ml) in phosphate buffer pH 7.0, vortexes, and incubated at 37°C for 2 h. The mucin-particle dispersions were centrifuged at 4,000 rpm for 2 min, and the supernatant was used for the measurement of the free mucin content by Lowry protein assay [16].

2.3.6.2 Ex vivo mucoadhesion experiment Ex vivo mucoadhesion studies were performed on freshly excised rat intestinal mucosa according to a method described previously [17]. Excised jejunum portion of the rat intestine was flushed with normal saline to remove luminal contents. Around 10 cm of intestinal tissue was cut opened and placed in a polyethylene support with help of cyanoacrylate adhesive. A 25 mg of samples were uniformly spread on the mucosal surface and were allowed to interact with mucus gel layer for 10 min. Tissue was then mounted on a platform at an angle 45° and washed under a constant flow rate (10 ml/min) of phosphate buffer (20 mM, pH 7.0). Particles washed from the mucosal surface were collected and dry weight was assessed. The weight of the dried particles was compared with the weight of the particles applied to the mucosal surface.

2.3.7 Cytotoxicity studies

The mouse fibroblast cell lines L-929 was obtained from the National Centre for Cell Science (NCCS, India). The cells were grown in Modified Eagle Medium (MEM, Sigma) containing 10% v/v Fetal Bovine Serum (FBS, Gibco). The cells were maintained at 37°C in an atmosphere of 95% air and 5% CO_2 at 90% relative humidity. Cells were trypsinized once per week with 0.05% Trypsin-EDTA solution (Sigma).

2.3.7.1 Cytotoxicity assay L-929 cells used for MTT assay was seeded on 24 well culture plates at seeding density of 5×10^5 cells per well in DMEM culture medium. The cells were grown in an atmosphere of 95% air, 5% CO_2 at 37°C and 90% humidity until continuous monolayer was obtained. Subsequently, culture medium was replaced with CS and CS derivatives and cells were then incubated with particles at 37°C for 24 h. The medium containing samples were removed and 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 reaction product was then solubilized in 500 µl of DMSO before quantifying the colour of reaction product using a microplate reader at 570 nm (Finstruments Microplate Reader). The cells treated with medium were used as negative control, while 10% phenol treated cells were used as a positive control. Experiments were carried out in triplicate

$$\% \text{ of viability} = \frac{\text{mean absorbance of sample}}{\text{mean absorbance of control}} \times 100$$

2.3.8 In vitro drug release study

2.3.8.1 Insulin release studies Insulin loading into the particles was achieved by a diffusion filling method. A known amount of dried particles (OC and LC) was soaked in insulin solutions for remote loading process and after defined time interval (6 h), loaded particles were taken out and dried under vacuum. For the release experiments, particles loaded with insulin were suspended in HCl–KCl buffer (0.2 M, pH 1.2) and phosphate buffer (0.2 M, pH 7.4). At specified intervals of time, aliquot of sample (200 µl) was withdrawn and insulin content was estimated by Lowry protein assay. The dissolution medium was replaced with fresh buffer to maintain total volume after each withdrawal.

2.3.8.2 Curcumin release Curcumin loading into the particles was achieved as follows. A known amount of dried particles (OC and LC) was soaked in curcumin which was dissolved in acetone for loading process. For the release experiments, particles loaded with curcumin were suspended in HCl-KCl buffer (0.2 M, pH 1.2) and phosphate buffer (0.2 M, pH 7.4). At specified intervals of time, supernatant solution was withdrawn and drug content was estimated by UV absorbance assay at 423 nm. The dissolution medium was replaced with fresh buffer after each withdrawal. The results are expressed as a percentage of drugs released.

3 Results and discussions

3.1 Synthesis and characterisations of derivatives using FTIR

Hydrophobic acylated CS derivatives were prepared by the modification of CS by acid chloride route using hexanoyl, lauroyl and oleoyl chlorides. The substitution on CS was confirmed by FTIR spectroscopy. FTIR spectrum of native CS, hexanoyl, lauroyl and oleoyl substituted CS is shown in Fig. 1.

FTIR spectra confirmed the acyl modification on CS. The peaks at 1,570 cm^{-1} can be assigned to the N–H bending vibrations of non-acylated primary amines in CS. After acylation, the vibrational band corresponding to the primary amino group at 1,570 cm^{-1} disappeared and the peak at 1,648 cm^{-1} appeared which is assigned to the

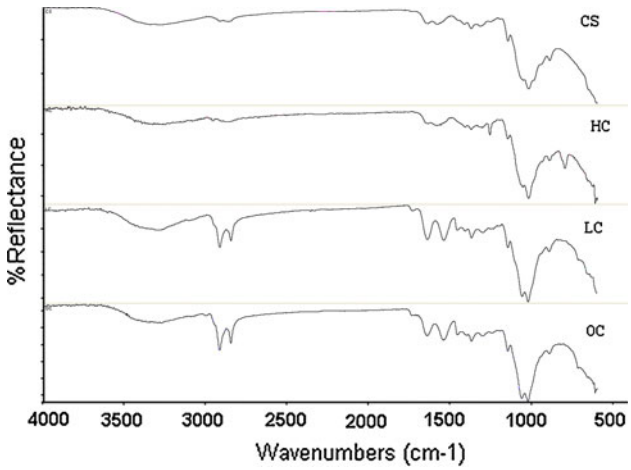

Fig. 1 FTIR spectra of CS (chitosan), HC (hexanoyl chitosan), LC (lauroyl chitosan), OC (oleoyl chitosan)

carbonyl stretching of secondary amide (amide I band). This shows the presence of amide bond in the modified CS. No peak was observed at 1,730 cm^{-1} which is the characteristic peak of carbonyl group of ester, showing that no substitution occurred on the hydroxyl group of CS. This further confirms the N-acylation on CS. The band at 2,920 cm^{-1} relates to $–CH_2$ vibration and the intensity of the band increased with increase in $–CH_2$ substitution. Reaction mechanism of acylation is shown in Scheme 1.

3.2 Particle size and zeta potential

Particle size and zeta potential of the CS derivatives was also affected by the acyl substitution. The difference in particle size and zeta potential is tabulated in Table 1. Zeta potential and particle size of native CS and CS derivatives further confirmed the modification process. The zeta potential of CS was decreased after the acylation process, which may be a result of the increase in the substituted groups. This indicates that the positive charge on the amino group of CS was reduced by the substitution with acyl groups, which in turn decreased the zeta potential of resultant CS derivatives.

Increase in particles size was also observed with the acyl modification process. However with increase in hydrophobicity, decrease in particle size was observed. The mean size distribution was inversely related to the hydrophobicity of the substituted groups in the polymer matrix. Tight packing of the hydrophobic components may reduce the particle size of the resultant micro structures.

3.3 TNBS assay

The degree of substitution was determined using trinitrobenzenesulphonic acid (TNBS) assay and is given in the Table 2.

3.4 Swelling studies

Figure 2 shows the swelling of CS and its derivatives (HC, LC & OC) at pH 7.4. As expected, the swelling of the CS particles was decreased with increase in their hydrophobic character. From the figure it was obvious that the swelling percentage of CS was decreased with increase in the acyl groups. This may be due to the addition of hydrophobic

Scheme 1 Reaction mechanism of acylation of chitosan

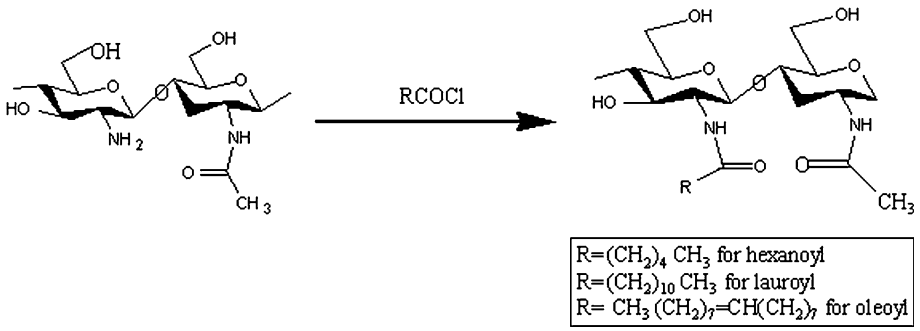

Table 1 Particle size and zeta potential of CS and acyl derivatives where *CS* chitosan, *HC* hexanoyl chitosan, *LC* lauroyl chitosan, *OC* oleoyl chitosan

Sample	Particle size (dnm)	Zeta potential (mV)		
		pH 1.2	pH 6.8	pH 7.4
CS	395.6 ± 62.48	46.4 ± 0.829	6.53 ± 0.325	3.91 ± 0.525
HC	780.4 ± 63.37	36.6 ± 1.28	2.88 ± 0.542	−4.68 ± 0.429
LC	564.2 ± 14.46	27.4 ± 0.417	4.85 ± 0.269	−7.44 ± 1.11
OC	457.3 ± 9.297	25.8 ± 0.795	−3.72 ± 0.468	−11.9 ± 1.87

Table 2 The percentage of free amino group and degree of substitution of CS and acyl derivatives where *CS* chitosan, *HC* hexanoyl chitosan, *LC* lauroyl chitosan, *OC* oleoyl chitosan

Sample	% Amino group	% Substitution
CS	66.5220	0
HC	58.5484	7.9736
LC	51.6116	14.9104
OC	49.2806	17.2414

group onto hydrophilic $-NH_2$ and also due steric repulsion of attached acyl groups. Further, possible formation of hydrogen bond between $-NH_2$ group and fatty acid could also influence the swelling nature of the micro particles.

3.5 Mucoadhesion studies

3.5.1 Mucin binding study

Mucin binding capability of CS and hydrophobic derivatives were determined under static in vitro conditions. The free mucin concentration was determined after 2 h incubation at neutral pH. The amount of mucin adsorbed was determined from the free concentration of mucin before and after adsorption. Percentage of unabsorbed mucin in the supernatant is plotted on Fig. 3. Results showed that mucin was adsorbed more on the CS derivatives compared to the native CS; mucin binding was increased with increase in the hydrophobic character. The result indicates that adhesion of CS may increase with increase in the hydrophobicity of the polymer system.

3.5.2 Ex vivo mucoadhesion experiment

Percentage of adhesion of CS and CS derivatives on excised rat intestinal tissue is shown in Fig. 4. The adhesion experiments also revealed that the CS derivatives showed better mucoadhesion compared to native CS. Mucins contains two different residues for mucoadhesive interactions—the charged acidic groups on sialic acid and sulphonated residues, and the hydrophobic methyl groups on fucose residues. Native CS has free amino group and this may interact with negatively charged sulphonated groups of mucin. However the derivatives are hydrophobic in nature and replaces or reduce the positive charge on CS. Both adhesion studies shows an increase in adhesion of CS with acylation and this increment was a function of substituted acyl groups [18]. Thus it can be assumed that the hydrophobic domains in the polymer interact firmly with mucin's hydrophobic peptide backbone segments [19]. Hydrophobic–hydrophobic interaction in this manner results in an interfacial force that holds the particles close to the mucus layer of the epithelium.

The application of CS as a mucoadhesive delivery vehicle has attracted attention towards peroral drug delivery applications. CS and derivatives are known to enhance the transmucosal drug absorption by improving the permeability of epithelial tight junctions [20–22]. The present study showed that the adsorption of particles on to the rat small intestine tissue can be enhanced with the hydrophobic modification process. Based on these result lauroyl and oleoyl substituted CS was considered for release experiments.

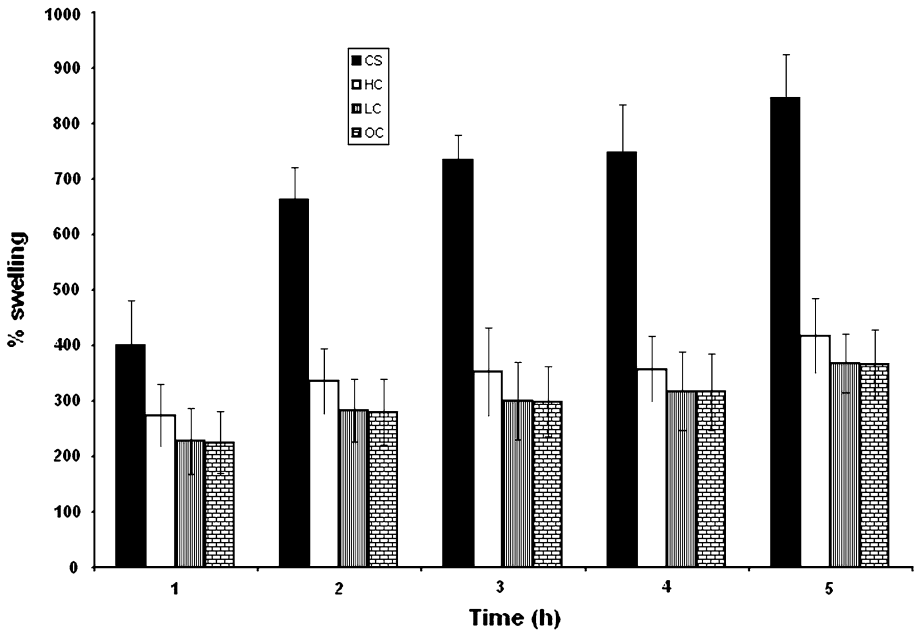

Fig. 2 Swelling studies of CS, HC, LC, and OC at pH 7.4

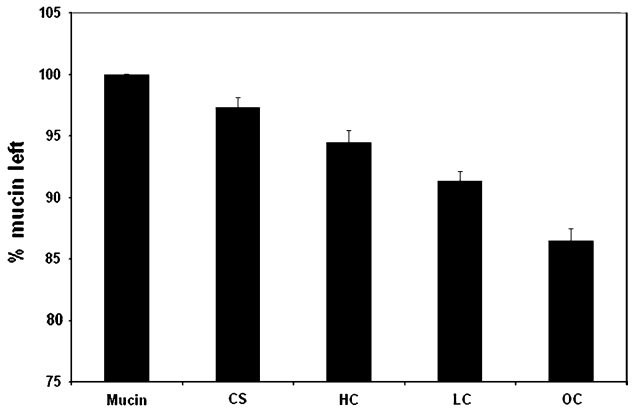

Fig. 3 Mucin binding studies of CS (chitosan) and acyl derivatives HC (hexanoyl chitosan), LC (lauroyl chitosan), OC (oleoyl chitosan)

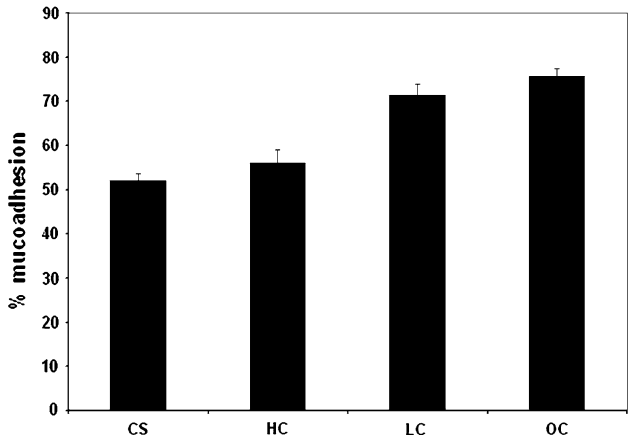

Fig. 4 Mucoadhesion studies of CS and acyl derivatives HC (hexanoyl chitosan), LC (lauroyl chitosan), OC (oleoyl chitosan)

3.6 Cytotoxicity studies

Percentage of viable fibroblast cells after contact with the polymer is shown in the Table 3. Cytotoxicity studies revealed that both native CS and acylated CS derivatives were non-toxic. L929 cell is an established cell line and which has been extensively used for cytotoxicity studies. The percentage of viable cells compared with control represented the level of cytotoxicity of the particles. The cleavage of MTT has desirable properties for assaying cell survival as MTT is cleaved by all living, metabolically active cells [23]. Percentage of viable cells for native CS was 92.5% and that of HC was 83.3%. Both OC and LC were found non-toxic as around 100% cell viability was obtained with these two systems. Nevertheless it can be concluded that the acyl derivatives of CS are non-toxic to the L-929 cells.

Table 3 Percentage of viable cells after cytotoxicity studies

Sample	% of viable cells
CS	92.56
HC	83.35
LC	100
OC	100

3.7 In vitro release experiments with LC and OC microparticles

3.7.1 Insulin release

The release of insulin from microparticles under gastric and intestinal pH conditions was investigated. Figure 5 illustrate the in vitro release of insulin from lauroyl CS (LC) and oleoyl CS (OC) derivatives. Release pattern was almost similar for both LC and OC; in both the case burst release of insulin was obtained at acidic and alkaline pH. Initial release was around 50% in gastric pH and about 60% was observed at intestinal pH. The observed release of insulin may be related to release from the micro particle surface, due to the weak interaction forces between the hydrophobic polymer and the protein. Insulin pattern of insulin was unaffected with the variation in the chain length of fatty acids attached to the CS polymer.

Acylated chitosan has been used widely used for drug delivery. Rekha and Sharma [22] developed lauryl succinyl modified chitosan nanoparticles for oral insulin delivery. About 60% insulin release was observed from the particles at pH 7.4 and showed sustained reduction in blood glucose level for 6 h, after an oral administration. Lauryl succinyl modified chitosan seems to be a promising matrix for the oral insulin delivery because of its capability of controlled delivery and enhanced mucoadhesivity compared to native chitosan. Self aggregating nanoparticles based hydrophobic anacardoylated chitosan was also used for developing oral

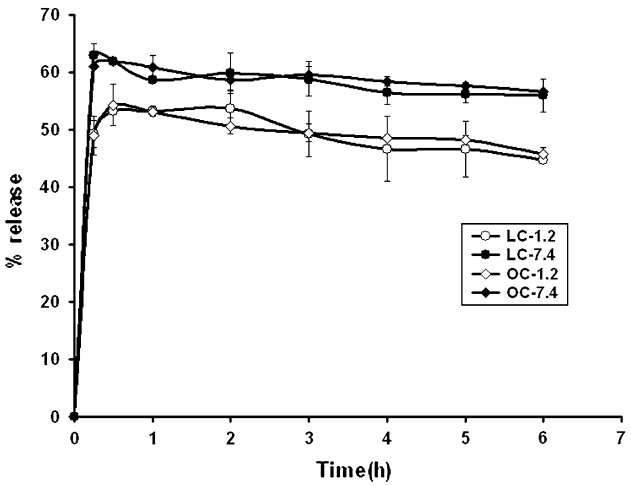

Fig. 5 Insulin release profile from LC and OC matrix

insulin delivery systems [24]. A moderately sustained release of insulin was observed from anacardoylated chitosan and it was also observed that the released insulin from the particles was stable and retained its conformation.

Du et al. [25] have studied the doxorubicin release from linoleic acid-grafted chitosan oligosaccharide (CSO-LA). The in vitro release studies indicated that the drug release from the DOX-loaded CSO-LA micelles was reduced with increasing the graft ratio of CSO-LA, due to the enhanced hydrophobic interaction between hydrophobic drug and hydrophobic segments of CSO-LA. Moreover, the drug release rate from CSO-LA micelles was faster with the drug loading.

3.7.2 Curcumin release

The release of curcumin was investigated to evaluate the release pattern of a hydrophobic compound from the fatty acid modified polymer. Figure 6 show the drug release behaviour of curcumin loaded lauroyl CS (LC) and oleoyl CS (OC) particles at gastric and intestinal pH medium as a function of time. The curcumin release from matrix was expressed by a plot of the fraction of pigment released at definite time period. Release pattern was almost similar for both LC and OC. In both the case, slow release was observed and sustained for 4 days. On the 4th day, the drug released was around 28 and 24% respectively from LC and OC particles at pH 1.2. However percentage of curcumin release from the same matrices at intestinal pH was 14 and 6% respectively. This indicated that these both matrices have a better sustained release property for curcumin, possibly due to their hydrophobic nature.

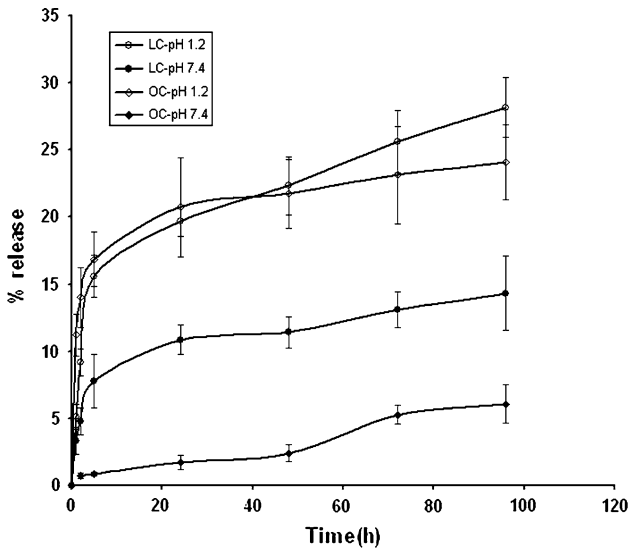

Fig. 6 Curcumin release profile from LC and OC matrix

In the case of hydrophilic drug, release was higher at intestinal pH but reverse was observed for a hydrophobic drugs. From both release studies it seems hydrophobic matrix are good choice for the encapsulation of hydrophobic compounds.

4 Conclusion

Hydrophobic interactions are believed to enhance the stability of substituted CS and are expected to be a promised model of drug delivery systems. In the study, acylated derivatives of CS were developed using various fatty acid chlorides. These systems displayed higher mucoadhesive interactions compared to the unmodified version and found non-toxic on fibroblast cell lines. Further release experiments conducted with two models drugs, insulin and curcumin. From the characterisation and subsequent studies, it was shown that these hydrophobic derivatives could be successfully used as the carrier for drug delivery system, particularly for hydrophobic drugs.

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