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Thiolated pectin nanoparticles: Preparation, characterization and *ex vivo* corneal permeation study

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ABSTRACT

The objective of present study was to prepare thiolated pectin nanoparticles and to evaluate them for ocular delivery. Thiolated pectin nanoparticles were prepared by ionotropic-gelation technique using magnesium chloride as the ionic cross-linker and timolol maleate as the model drug. The results revealed that increasing the concentration of magnesium chloride results in significant increase in particle size, while % entrapment is decreased significantly by increase in the concentration of thiolated pectin. The optimal formulation having particle size of 237 nm and % entrapment of 94.6% was obtained at concentrations of thiolated pectin – 0.01% (w/v) and magnesium chloride – 0.01% (w/v). On comparative evaluation, thiolated pectin nanoparticulate formulation provided significantly higher *ex vivo* corneal permeation of timolol maleate across the excised goat cornea than the conventional aqueous solution.

1. Introduction

Natural polysaccharides and their derivatives have been used extensively in pharmaceutical and food industry due to their biocompatibility, biodegradability and non-toxicity. These are used as thickening agents, gelling agents, emulsifying agents, binding agents, encapsulating agents, swelling agents, foam stabilizers, etc. (Ahuja, Yadav, & Kumar, 2010). Natural polysaccharides belong to the 'first generation' mucoadhesives. Their mucoadhesive properties depend upon the formation of hydrogen bonds between the hydroxyl, carboxyl and amine groups on the polysaccharide molecule with the mucosa. The non-covalent interaction between the polysaccharide and mucus layer results in weak mucoadhesion (Smart, 2005). Mucoadhesive properties of natural polymers can be improved by their thiolation. Thiol containing ligands can be introduced into polysaccharide chain with the formation of amide or ester bonds. Thiol side chain of thiolated polymers interacts with cysteine rich subdomains of mucus glycoprotein forming stronger disulfide bonds (covalent bonds) between mucoadhesive polymer and mucus layer (Bernkop-Schnürch, 2005). During earlier investigations thiolated chitosan (Bernkop-Schnürch, Hornof, & Zoidl, 2003), alginate (Bernkop-Schnürch, Kast, & Richter, 2001), gellan (Krauland, Leitner, & Bernkop-Schnürch, 2003), pectin (Perera, Hombach, & Bernkop-Schnürch, 2010; Sharma & Ahuja, 2011) have been prepared to improve their mucoadhesive properties.

Topical application of drugs to eye is the most accessible and preferred mode of treatment for management of ocular ailments. But it is limited by the shorter pre-corneal residence time of the drug, which requires frequent topical administration. To overcome this drawback, ocular inserts and collagen shields have been employed (Higashiyama, Inada, Ohtori, & Tojo, 2004). They prolong the contact time of the vehicle at ocular surface as well as slow down the elimination of the drug (Bourlais, Acar, Zia, Sado, Needham, & Leverge, 1998). But the difficulty in use of ocular inserts by the elderly people and their loss from the eyes without the patient becoming aware of it, are the major limitations, which have limited their widespread use (El-Kamel, 2002). Thus, taking into account the patient acceptability and ocular bioavailability, bioadhesive submicron particulate carriers formulated in liquid dosage form appear to be the most promising ocular delivery system.

The objective of present study was to formulate and optimize thiolated pectin nanoparticles. Timolol maleate, a beta-adrenergic blocker used widely for lowering the intraocular pressure of glaucoma patients, was used as the model drug. Timolol maleate-loaded thiolated pectin nanoparticles were prepared by ionotropic gelation method using magnesium chloride as cross-linking agent. The preparation of thiolated pectin nanoparticles was optimized using response surface methodology. Thiolated pectin nanoparticles were evaluated for particle size, shape, drug entrapment and *ex vivo* corneal permeation characteristics using isolated goat cornea.

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2. Experimental

2.1. Materials

Pectin (GENU®pectin (citrus) type USP/100, CP Kelco US, Inc.) was gifted by Burzin and Leons Agenturen Pvt. Ltd. (Mumbai, India). Timolol maleate was obtained as gift sample from Optica Pharmaceuticals (Yamunanagar, India). Magnesium chloride, thioglycolic acid and hydrochloric acid were purchased from SD Fine-Chem Ltd. (Mumbai, India). Freshly excised goat eye was obtained from the local butcher shop (Hisar, India).

2.2. Synthesis of thiolated pectin

Synthesis of thiolated pectin was done as reported earlier by esterification of pectin with thioglycolic acid in the presence of hydrochloric acid (Sharma & Ahuja, 2011). Briefly, pectin (16 g) was reacted with 80% thioglycolic acid (7.59 g) in acidic conditions for 150 min at 80 °C. Thiolated pectin was precipitated by adding the reaction mixture into methanol followed by washing with methanol and drying at room temperature. The synthesized thiolated pectin was found to have 0.60 ± 0.04 mmol of thiol groups/g of thiol group as determined by Ellman's method (Bernkop-Schnürch et al., 2003).

2.3. Preparation of thiolated pectinate nanoparticles

Timolol maleate-loaded thiolated pectin nanoparticles were prepared by ionotropic gelation method using magnesium chloride as cross-linking agent (Opanasopit, Apirakaramwong, Ngawhirunpat, Rojanarata, & Ruktanonchai, 2008). An aqueous solution of thiolated pectin (0.1–0.01%, w/v) containing timolol maleate (50%, w/w of thiolated pectin) was added drop by drop into aqueous solution of magnesium chloride (0.1–0.01%, w/v) with constant stirring at 20 °C.

2.4. Experimental design

Preparation of timolol maleate-loaded thiolated pectin nanoparticles was carried out employing a central composite design with $\alpha = 1$ as per the standard protocol. The concentration of thiolated pectin and concentration of magnesium chloride were selected as formulation variables on the basis of previous trials. The central point (0, 0) was studied in pentet. All other formulation and processing variables were kept invariant throughout the study. Table 1 summarizes an account of the 13 experimental runs studied, their factor combination and the translation of the coded levels to the experimental units employed during the study. Particle size and percentage entrapment were taken as response variables. The experimental design and statistical analysis of data were done using the Design Expert software (Version 8.0.4, Stat-Ease Inc., Minneapolis, MN).

2.5. Characterization of the thiolated pectinate nanoparticles

2.5.1. Particle size

The mean particle size of the drug loaded thiolated pectinate nanoparticles was determined at 25 °C by photon correlation spectroscopy (PCS) using the Zetasizer Nano ZS (Malvern Instruments, Malvern, UK).

2.5.2. Entrapment efficiency

The entrapment efficiency (%) of the timolol maleate-loaded thiolated pectin nanoparticles was determined indirectly upon separation of the nanoparticles by centrifugation at 15,000 rpm for 30 min from the aqueous medium containing free timolol maleate. The amount of free timolol maleate in the supernatant was determined spectrophotometrically by measuring absorbance at 294 nm. The entrapment efficiency of thiolated pectin nanoparticles was calculated as the ratio of timolol maleate loaded into the nanoparticles with respect to the total amount of timolol maleate used in the preparation of nanoparticles as follows:

$$\text{EE } (\%) = \frac{\text{TM}_t - \text{TM}_f}{\text{TM}_t} \times 100 \quad (1)$$

where TM_t is the total amount of timolol maleate used in the preparation of the nanoparticles and TM_f is the free timolol maleate present in the supernatant. All samples were measured in triplicate (Saremi, Atyabi, Akhlaghi, Ostad, & Dinarvand, 2011).

2.5.3. Morphology

The morphology of timolol maleate-loaded thiolated pectin nanoparticles was studied using transmission electron microscope (TEM; Hitachi H7500).

2.6. Preparation of timolol maleate ophthalmic formulations

2.6.1. Formulation of timolol maleate (0.005% w/v) ophthalmic nanosuspension

The optimized timolol maleate-loaded thiolated nanoparticle formulation was adjusted to pH 7 with sodium hydroxide and made isotonic by adding the required amounts of sodium chloride as tonicity modifier.

2.6.2. Formulation of timolol maleate (0.005% w/v) ophthalmic solution

Required quantities of timolol maleate was dissolved in magnesium chloride solution, adjusted to pH 7 and made isotonic by adding the required amounts of sodium chloride as tonicity modifier.

2.7. Ex vivo corneal permeation

Timolol maleate-loaded thiolated pectinate nanoparticles were evaluated for corneal permeation characteristics using the isolated goat cornea model (Yadav & Ahuja, 2010). Freshly excised goat eyeballs were obtained from a local butcher house (Hisar, India) within an hour of slaughter, and transported to laboratory in cold isotonic saline. Cornea was excised along with 2–4 mm of scleral tissue, and washed with cold normal saline to remove the adhering tissue and proteins. Isolated cornea was clamped between the donor and receptor cells of the modified Franz diffusion cell in such a way that epithelial side faced the donor and endothelial side faced the receptor cell. The receptor cell comprised of 11 ml of freshly prepared Ringer bicarbonate under magnetic stirring and maintained at 35 ± 1 °C by circulating water in the water jacket from the thermostated water bath. One milliliter of the test formulation was placed over the cornea. An aliquot of 2 ml sample was withdrawn at the end of 2 h and analyzed for the contents of timolol maleate spectrophotometrically at 294 nm. Corneal hydration was determined at the end of the experiment to check the corneal integrity. Cornea was freed from the scleral tissue and weighed, followed by overnight soaking in methanol and drying in an oven at 90 °C and weighing again.

3. Results and discussion

The covalent attachment of pectin to thioglycolic acid was achieved by ester bonds formation between hydroxyl group of galacturonic acid moieties of pectin and carboxyl group of thioglycolic acid. After being ground in a mortar, product appeared as off-white odorless powder, which is soluble in water.

Table 1
Central composite design using formulation variable influencing particle size (Y_1) and percentage entrapment (Y_2).

Exp. no.	Concentrations (%, w/v)		Particle size (nm) (Y_1)	Entrapment (%) (Y_2)
	Thiolated pectin (X_1)	Magnesium chloride (X_2)		
1	0.01(−1)	0.10(+1)	593	84.07
2	0.06(0)	0.06(0)	632.5	18.04
3	0.10(+1)	0.06(0)	463	7.86
4	0.06(0)	0.10(+1)	634.4	18.2
5	0.06(0)	0.06(0)	665.3	15.72
6	0.10(+1)	0.10(+1)	614.5	1.82
7	0.10(+1)	0.01(−1)	155	9.56
8	0.01(−1)	0.06(0)	632	92.13
9	0.06(0)	0.06(0)	820	15.16
10	0.06(0)	0.06(0)	692	15.1
11	0.06(0)	0.01(−1)	408	18.48
12	0.06(0)	0.06(0)	654.8	18.6
13	0.01(−1)	0.01(−1)	194	85

The negatively charged, carboxyl groups of pectin crosslink intermolecularly with divalent cations forming gel ("egg-box model"). This reaction has been used frequently for preparing ionotropically gelled beads of pectin (Aydin & Akbug, 1996; Sriamornsak, 1999). This laboratory earlier reported formulation of ionotropically gelled thiolated pectin beads and exploited its mucoadhesive application employing metformin as a model drug (Sharma & Ahuja, 2011). In the present investigation, ionotropic gelation of thiolated pectin by Mg^{2+} has been utilized to prepare the nanoparticulate formulation, as Mg^{2+} ions were earlier reported to provide smaller size particles of pectin (Opanasopit et al., 2008).

The preparation of thiolated pectinate nanoparticles was optimized using response surface methodology employing the central composite design (Table 1). The results of response generated using design were fitted into polynomial models and ANOVA test was applied to models to estimate their significance. The results of this analysis revealed that percentage entrapment (Y_2) fitted best into the response surface quadratic model while the response particle size (Y_1) fitted best into the response surface quadratic model with backward elimination.

The polynomial models for response Y_1 and Y_2 can be represented by Eqs. (2) and (3) respectively.

$$Y_1 = 691.08 - 31.08X_1 + 180.82X_2 - 138.98X_1^2 - 165.28X_2^2 \quad (2)$$

$$Y_2 = 17.44 - 40.36X_1 - 1.49X_2 - 1.70X_1X_2 + 30.19X_1^2 - 1.37X_2^2 \quad (3)$$

The polynomial equations comprise the coefficient for intercept, first-order main effect, interaction terms, and high order effects. The sign and magnitude of main effect signify the relative influence of each factor on the response. A negative sign signifies an antagonist effect while a positive sign indicates a synergistic effect.

Table 2 represents the results of ANOVA test on the quadratic regression models, which indicate that response surface model developed for the two responses were significant and adequate, without significant lack of fit. The results of model summary statistics show the R^2 value >0.9, for both the response models, which indicate a good correlation between the experimental and predicted responses. In addition, the predicted R^2 value is in reasonable good agreement with adjusted R^2 value, indicating reliability of models. Further, the higher values (>4) of "Adequate Precision" indicate adequate signal. The relatively lower values of coefficient of variation indicate better precision and reliability of the experiments.

Table 3 represents the results of factor effects and associated p-values for responses Y_1 and Y_2. The data reveals that significant factors affecting the response Y_1 were the synergistic effects of linear contribution of X_2, while the quadratic contribution of X_1 and X_2 antagonistically affected Y_1. The response Y_2 was significantly affected by antagonistic effect of linear contribution of X_1 and synergistic effect of quadratic contribution of X_1.

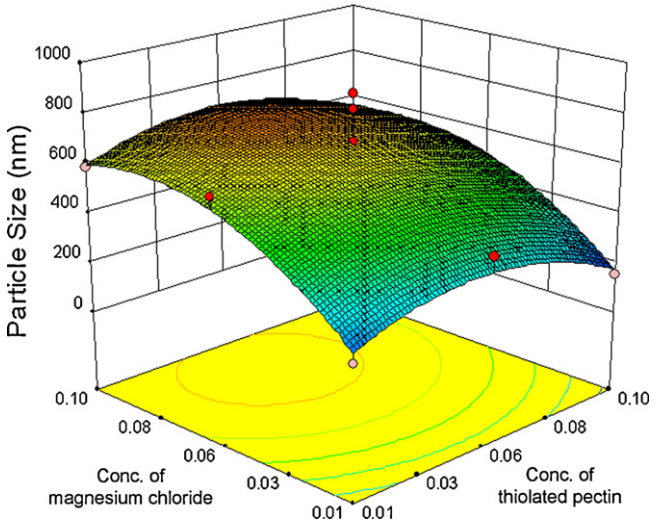

Fig. 1. Response surface plot showing the combined effect of concentrations of thiolated pectin and magnesium chloride on the particle size of nanoparticles.

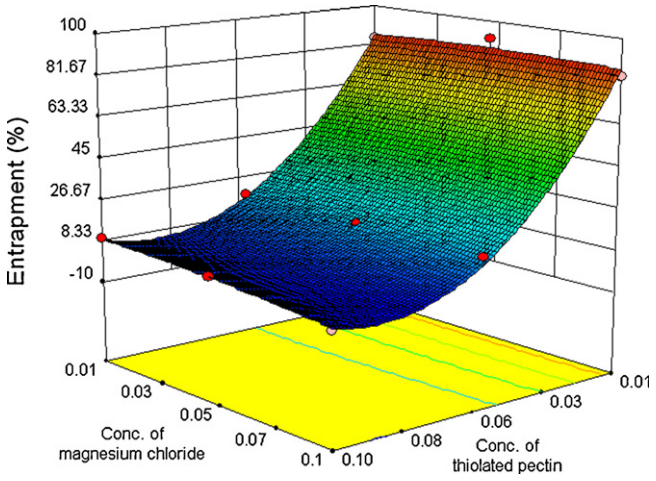

Fig. 2. Response surface plot showing the combined effect of concentrations of thiolated pectin and magnesium chloride on the percentage entrapment of drug in nanoparticles.

Table 2
Model summary statistics.

Response factor	Model						Lack of fit	
	F-value	Prob > *F*	R^2	Pred. R^2	Adeq precision	C.V.	*F*-value	Prob > *F*
Particle size (Y_1)	18.05	0.0005	0.9003	0.7137	11.389	13.67	1.06	0.4785
Entrapment (%) (Y_2)	309.43	<0.0001	0.9955	0.9686	43.908	9.3	5.51	0.0663

Table 3
Summary of each factor effect and its *P*-value.

Factor	Y_1		Y_2	
	Factor effect	*P*-value	Factor effect	*P*-value
X_1	−31.08	0.3414	−40.36	<0.0001
X_2	180.82	0.0004	−1.49	0.2420
X_1^2	−138.98	0.0154	30.19	<0.0001
X_2^2	−165.28	0.0065	−1.37	0.4506
X_1X_2	–	–	−1.70	0.2725

Figs. 1 and 2 portray the 3-D response surface plots constructed using the models generated by response surface methodology. Fig. 1 shows the combined effect of concentrations of thiolated pectin and magnesium chloride on the particle size of nanoparticles. It can be observed from the plots that concentration of magnesium chloride has more pronounced effect on particle size of nanoparticles than concentration of thiolated pectin. As the concentration of magnesium chloride is increased from 0.01 to 0.1% (w/v), the particle size is increased correspondingly from 155 to 820 nm. Earlier investigation with pectinate micro/nanoparticles, also revealed that decreasing the concentration of pectin and/or divalent cations provided particles with smaller particle size (Opanasopit et al., 2008). In a study of calcium-pectinate beads, the augmentation of particle size with increase in the concentration of Ca^{2+} was attributed to the increased retention of Ca^{2+} leading to higher water retention (Das & Ng, 2010). Ionically gelled polysaccharide nanoparticles are formed in very dilute solutions using the concentration of gelling agent below the gel-point i.e., pre-gel phase, where the polymeric chains react with gelling agent to form small clusters (Vauthier & Bouchemal, 2009). The weaker interaction between thiolated pectin and Mg^{2+} ions at the lower cross-linker concentration corresponding to the pre-gel phase might be responsible for formation of smaller size nanoparticles. Thus, increasing the concentration of cross-linking ion above the gel point will result in bigger size particles. The gelling ability of pectins with divalent cations is reported to follow the order – $Mg^{2+} \ll Ca^{2+}$, $Sr^{2+} < Ba^{2+}$ (Racovita, Vasiliu, Popa, & Luca, 2009). This could be one of the reasons for the smaller size of pectinate nanoparticles obtained with use of magnesium compared to the calcium, as a gelling counter ion (Opanasopit et al., 2008).

Fig. 2 shows the effect of concentrations of thiolated pectin and magnesium chloride on the percentage entrapment of drug in nanoparticles. It can be observed that concentration of thiolated pectin has more pronounced effect on % entrapment than the magnesium chloride concentration. The decrease in % entrapment with the increase in thiolated pectin concentration can be attributed to the inadequate interaction between the thiolated pectin and magnesium chloride because of increase in viscosity of solutions with increase in thiolated pectin concentration.

A numerical optimization technique using the desirability approach was employed to develop a new formulation with the desired responses. The optimization was done with constraints for minimum particle size (Y_1), and maximum percentage entrapment (Y_2) as the goals to locate the optimum setting of independent variables in the new formulation. The optimization tool provided us with six sets of possible solutions. The optimal calculated parameters with highest desirability were concentrations of thiolated pectin – 0.01% (w/v) and magnesium chloride – 0.01% (w/v). Using these parameters a batch of timolol maleate-loaded thiolated pectin nanoparticles was prepared, which was found to have the particle size (Y_1) of 225 nm (predicted 237.08 nm), and % entrapment efficiency (Y_2) of 94.6% (predicted 86.39%). The lower values of % prediction error (−5.36% for Y_1 and 8.67% for Y_2) indicate the reliability of developed mathematical models.

Fig. 3 shows transmission electron micrographs of timolol maleate-loaded thiolated pectin nanoparticles. The nanoparticles appear to be of ovoid morphology. Since the timolol maleate-loaded thiolated pectin particles are of nanometric ranges and ovoid in morphology, they are not expected to cause an irritation.

Table 4 compares the results of *ex vivo* corneal permeation of timolol maleate from the thiolated pectin nanoparticle suspension with the conventional ophthalmic solution across the isolated goat cornea. The results reveal significantly higher corneal

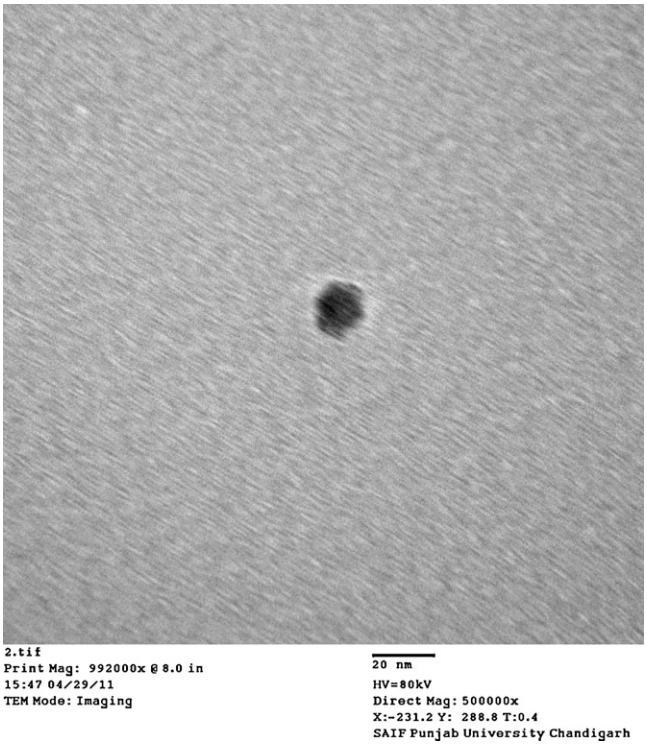

Fig. 3. Transmission electron micrographs of timolol maleate-loaded thiolated pectin nanoparticle.

Table 4
Ex vivo corneal permeation of timolol maleate from timolol maleate-loaded thiolated pectin nanosuspension (0.005%, w/v) and timolol maleate ophthalmic solution (0.005%, w/v).

Formulation	Corneal permeation[a] (%)	Hydration[a] (%)
Ophthalmic nanosuspension	6.23 ± 1.50	75.12 ± 3.28
Ophthalmic solution	1.78 ± 0.38	78.16 ± 2.11

[a] Values are mean ± sd (n = 3).

permeation of timolol maleate from the formulation based on thiolated pectin nanosuspension compared to the conventional formulation based on aqueous solution. The corneal hydration levels are indicative of corneal integrity and its normal levels are reported to be 75–80% (Maurice & Riley, 1970). As the corneal hydration levels in the present study are within limits indicating integrity of corneal epithelium and endothelium. During earlier studies drugs formulated as nanoparticulate carriers have been reported to provide a higher corneal permeation which was attributed to the endocytic uptake (Calvo, Vila-Jato, & Alonso, 1996; Gupta, Madan, Majumdar, & Maitra, 2000). Thiolated polymers have also been reported to exhibit permeation-enhancing effect (Di Colo, Zambito, & Zaino, 2008). Thus, enhanced permeation of timolol maleate across excised goat cornea from thiolated pectin nanosuspension may be attributed to the endocytic uptake and/or permeation enhancing effect of thiolated polymers. However, further studies are needed to comment more on this aspect.

4. Conclusion

The preparation of timolol maleate-loaded thiolated pectin nanoparticles was optimized by response surface methodology using central composite design. The concentration of cross-linker was observed to exert more pronounced effect on particle size of nanoparticles, while concentration of polymer showed prominent effect on drug entrapment. The optimal formulation of nanoparticles showed significantly higher *ex vivo* corneal permeation compared to the conventional solution dosage form. Further, the mucoadhesive nanoparticles are expected to provide a release of drug over the prolonged duration from the particles lodged in the cul-de-sac. On the basis of this study it can be concluded that thiolated pectin is promising mucoadhesive polymer for ocular delivery of timolol maleate.

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