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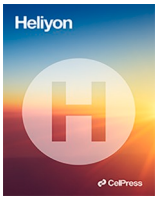

Research article

Quantitative analysis of loxoprofen sodium loaded microspheres comprising pectin and its thiolated conjugates: *In-vivo* evaluation of their sustained release behavior

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ABSTRACT

Continuous use of oral NSAIDs can damage mucosal membrane, which results in decreased bioavailability and non-compliance with the therapy. But the use of sustained release drug delivery systems might offer a solution. Objective was to synthesize mucoadhesive SR microspheres by using different combinations of pectin (PEC) and its thiolated derivative (T-PEC3100) for improved loxoprofen (LS) permeation. Thiolated pectin (T-PEC) was synthesized by the esterification method using thioglycolic acid. Thiolation was confirmed by thiol group quantification and charring point determination. Further characterization was done by Fourier Transform Infrared spectroscopy (FTIR), and Scanning electron microscopy (SEM). Ex-vivo mucoadhesion study was performed to confirm the improved characteristics. Microspheres (MS) were prepared using different ratios of PEC/T-PEC by solvent evaporation method and their particle size and surface morphology were evaluated. Mucus permeation study was carried out using the trans-well plate method. Sustained release behavior of prepared microspheres was investigated through the edema inhibition method in albino rats. T-PEC3100 was considered the optimum formulation for further evaluation and contained maximum thiol group content. FTIR spectra showed a characteristic peak of –SH and charring point was also changed considerably confirming the successful thiolation of PEC. SEM results showed spherical microspheres in the size range of 2–10 μm. Thiol-rich formulation of MS exhibited more than 80 % release after 12 h and maximum absorbable dose (MAD) was calculated as 400 μg % inhibition of edema in MS treated group was slowly attained initially but the reduction in inflammation was detected even after 24 h as compared to

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control group. Promising results from *In-vivo* edema inhibition study suggest the possible use of these thiolated MS in formulating sustained release formulation for arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease that is characterized by persistent joint inflammation which ultimately results in severe disability and premature mortality [1]. The global prevalence of RA is 1 % and the risk is 2–3 times greater in women than men. In the pathogenesis of arthritis chronic inflammation, the synovial membrane leads to bone erosion, destruction of articular cartilage, and permanent deformities. Disease-Modifying Antirheumatic Drugs (DMARDs), multiple dosing therapies of NSAIDs, corticosteroids, and biologicals are the available options to treat RA. NSAIDs are widely accepted as analgesics and anti-inflammatory drugs for the treatment of such conditions. But, in multiple doses, these drugs produce gastric and intestinal epithelium erosions and longitudinal ulcers, which lead to non-compliance with the treatment. Loxoprofen sodium (LS) is a propionic acid derivative, which has been used for the treatment of Rheumatoid arthritis as well as for Osteoarthritis, postoperative pain, and toothache. Because of its shorter half-life, it has to be administered three times a day, which may cause gastric mucosal lesions. Therefore, it's a major cause of patient non-compliance in patients suffering from chronic arthritis. Additionally, conventional drug delivery systems give initial burst release of LS owing to its high aqueous solubility, which is also a cause of mucosal injury [2].

Oral Sustained release formulation is one of the choices for chronic diseases like RA because of their ease of administration, patient compliance, and improved bioavailability. But one of the concerns associated with these systems is their rapid clearance from GIT which results in incomplete drug release and low bioavailability [3]. Many techniques have been investigated to increase the residence time of delivery systems and thiolation came out as a better option than other grafting techniques. Since, the introduction of thiomers, several thiolated polymers have been reported to provide enhanced residence time, improved efflux pump inhibition effect, and controlled drug [4,5]. The introduction of thiol group into pectin backbone was quite a simple one step process. The mechanism involved was esterification, in which hydroxyl group of galacturonic acid in pectin reacted with carboxylic group of thioglycolic acid. The covalent bond formation between two functional group resulted in the attachment of –SH group into pectin. The thiol group along the side chains of the polymer reacts with the cysteine-rich sub-domains of the mucous membrane and forms a disulfide bond which is much stronger than hydrogen bonding and ionic interactions of polymers [6,7]. Pectin is a naturally found heteropolysaccharide, which has found numerous applications in the food and pharmaceutical industry because of its biodegradable and non-toxic nature [8].

Pectin is a naturally found heteropolysaccharide, which has found numerous applications in the food and pharmaceutical industry because of its versatile chemical structure, selective solubility and non-toxic nature. An important attribute of pectin, is its mucoadhesive nature, owing to its anionic nature, which makes the formulation reside at the absorption site for longer than usual and render it a useful candidate for sustained release formulation. The basis of the mucoadhesive property is the presence of hydroxyl groups in its structure [9], which make strong covalent bonds with the glycoproteins of mucin [10]. Based on the chemical structure of pectin, it is composed of α -galacturonic acid with varying degrees of acetylation and methylation, which affects its interaction with various drugs such as encapsulation efficiency release and stability of delivery systems. Considering solubility, Pectin is soluble in water, and forms gels in the presence of divalent cations. Thus, the swelling behavior in response to environmental factors also affects the pharmacokinetics properties. Most importantly, Pectin has already some degree of mucoadhesive potential which is due to the interaction of hydroxyl group of Pectin and hydroxyl group present in mucus. Moreover, their biocompatible natures along its bio-adhesion make it a good option for targeting release of drugs. Self-assembling, pectin-based liposomes containing calcitonin were prepared and evaluated for the improvement in mucoadhesive strength. The results were quite promising and suggested that low methoxy pectin could prolong the retention time of multiple unit dosage forms [11].

Additionally, after being grafted with thiol group, thiolated pectin, have been reported to impart enhanced mucoadhesive property to the delivery systems [12]. Sharma et al. prepared thiolated pectin nanoparticles to improve the corneal residence time and permeation of the drug and reported 2X increase in mucoadhesion time [13]. Similarly, in a recent studies, thiolated pectin was bridged with thiolated eudragit into nanoparticles for the co-delivery of aspirin and metformin in the treatment of colorectal cancer [14,15]. The pH sensitive nanoparticles formulation exhibited targeted drug delivery of the drugs as exhibited by the pharmacokinetics data. The pharmacokinetic data shows enhanced $AUC_{0\text{-}t}$ and mean residence time of aspirin and metformin from the optimized nanoparticle formulation in comparison with the reference which declare these formulations as sustained release. Microparticles, depending upon many factors e.g., size and of drug molecules, concentration of surfactants or polymers, are being used efficiently to encapsulate a number of drugs [16–18].

The present study aimed to increase the mucoadhesion strength of low methoxy PEC by synthesizing its thiolated derivative. The effect of thioglycolic acid (TGA) concentrations used in the reaction was evaluated on the duration of mucoadhesion and thiol content of T-PEC. *Ex-vivo* mucoadhesion study was performed to validate the improved mucoadhesive characteristic. Both polymers were characterized by Fourier Transform Infrared spectroscopy (FTIR), Scanning electron microscopy (SEM), Differential scanning calorimetry (DSC), and X-Ray diffraction (XRD) for evaluating the differences. Optimization of formulations and preparation of their MS by using different ratios of low methoxy PEC and T-PEC3100 widened new horizons for the improvement of sustained release drug delivery systems. Results of *Ex-vivo* permeation studies by trans-well cells and the *in-vivo* anti-inflammatory studies by rat paw edema method further established their use in arthritis patients.

2. Materials and methods

2.1. Materials

Pectin (PEC, M.W = 194.14), thioglycolic acid (TGA, M.W = 92.12), and Ellman's reagent (5, 5′-dithiol, bis-2-nitro benzoic acid) were purchased from Sigma Aldrich (M.W = 396.35, Germany). Methanol (CH3OH, M.W = 32.042) was purchased from POCH (Poland) and hydrochloric acid (HCl, M.W = 36.46) was purchased from Fluka (Germany). Loxoprofen sodium was gifted by SAJA Pharmaceuticals Pakistan. Sodium hydroxide (NaOH) and potassium dihydrogen phosphate (KH2PO4) were purchased from Merck, Germany. All chemicals were of analytical grade, double distilled and deionized water was used for the whole study.

2.2. Synthesis of T-PEC

T-PEC was synthesized by the esterification of hydroxyl groups of low methoxy PEC by slight modifications of the previously reported method of Dicharry et al., [19]. The reaction scheme is given in Fig. 1. Briefly, an aqueous suspension of PEC and TGA was prepared in different molar concentrations (1:1, 1:2, 1:3, 1:4, 1:5) in de-ionized water at room temperature with continuous stirring at 1500 rpm. For the acidification of the mixture freshly prepared 7N, HCl was added dropwise, and the mixture was kept under continuous stirring to react completely at 80 °C for 3 h. Finally, the resultant mixture was added to 200 mL methanol and continuously stirred until precipitation. The obtained precipitates were washed 3 times with methanol, 2 times with double distilled water, dried overnight at room temperature first, then in an oven at 40 °C, and stored in a desiccator for further use [20]. The percentage yield of all formulations was calculated.

2.3. Determination of thiol content and charring point of T-PEC

For quantification of thiol group immobilized on the PEC, previously described method was used with slight modifications [10]. Ellman's test was performed by hydrating 1 mg sample of T-PEC in 0.5 mL buffer (0.5 M phosphate buffer pH 8). 10 mL freshly prepared Ellman's reagent (0.03 %) of 5, 5-dithiobis 2-nitrobenzoic acid) was dissolved in buffer and pH 8 was adjusted by using 0.2 M NaOH. After that, 2 mL of Ellman's reagent was added to the reaction mixture and incubated. After 3 h of incubation in dark at room temperature, 200 μL solution was analyzed using spectrophotometer for thiol content at 450 nm. Same procedure was conducted without adding thioglycolic acid for the comparison. Melting point apparatus (Stuart SMP3, England) was used to determine the charring point of PEC and all formulations of T-PEC. The temperature was increased to 10 °C/min until all samples were completely charred [21]. Physical changes were observed in each sample and results of the mean ± SD (n = 3) were reported.

2.4. Characterization of T-PEC

Morphology of PEC and T-PEC3100 was observed by using Hitachi High Tech S4800 FE-SEM. Samples were coated with gold in a dry state and placed on a sample holder at an accelerating voltage of 15 kV. FTIR was performed (400-4000 cm^{-1}) for the detection of functional groups of both polymers (PEC, T-PEC3100) by ATR-FTIR (Bruker Alpha I, Germany) for the identification of –OH, –COOH, and –SH functional groups in PEC and T-PEC respectively [22]. DSC analysis was performed to detect any change in the thermal properties of PEC after thiolation by using DSC (PerkinElmer, 60A, Germany). Briefly; 6 mg of both polymers were heated in an aluminum pan over a temperature range of 10–400 °C under a nitrogen atmosphere at 20 mL/min. The temperature was increased gradually at the rate of 10 °C/min. X-ray diffractometry was carried out by using an X-ray diffractometer (D/max-2500pc, Rigaku. Co, Japan) operating in the reflection mode (35 kV, 30 mA) to evaluate the difference in crystallinity of PEC and T-PEC3100. Angle 2Θ in the range of 0-80° within applied voltage of 35 (kV) with scan running speed 0.050/min were the experimental conditions.

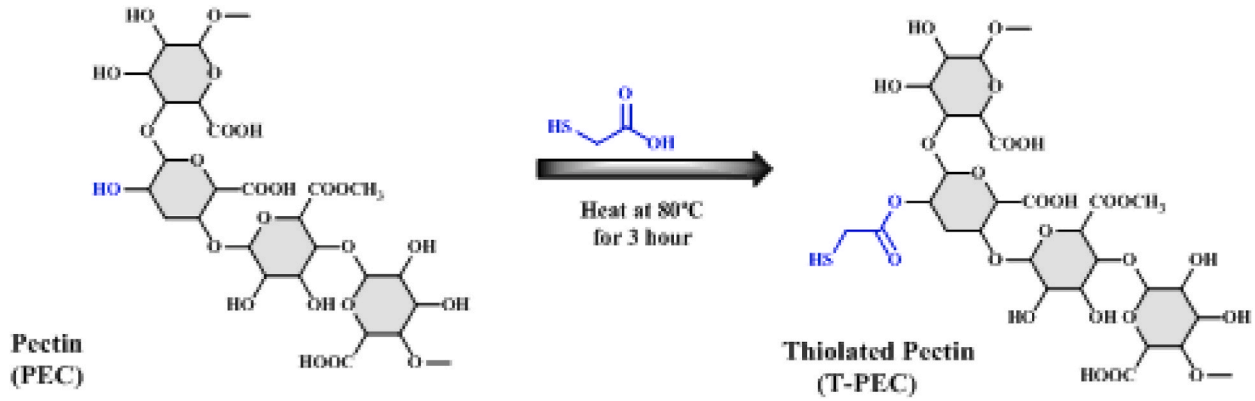

Fig. 1. Schematic illustration of PEC reacting with thioglycolic acid to synthesize T-PEC.

2.5. Ex-vivo mucoadhesion and swelling evaluation

Ex-vivo mucoadhesion assay was performed by using basket rack assembly. Briefly, the freshly excised rabbit intestine was obtained from a local butcher, washed with normal saline and attached to a modified USP I disintegration apparatus (Erweka, GmBH, Germany) using cyanoacrylate adhesive. Accurately weighed 20 mg PEC and T-PEC were compressed into 5 mm thick flat discs and adhered to the mucosal membrane by applying slight pressure for proper adhering. The basket was immersed in phosphate buffer and operated at 0.5 dips per second (DPS) at 37 °C. The detachment time of PEC and T-PEC discs was noted visually [23]. Similarly, the swelling behavior of the compressed disc of PEC and T-PEC was studied by immersing it in 100 mM phosphate buffer of pH 1.2 and 6.8. The swelled discs were removed, blotted on filter paper, and weighed until constant. The % swelling was then calculated by using the following equation.

$$Q(\%) = \frac{M_s - M_d}{M_d} \times 100 \quad (1)$$

where M_s, and M_d are the swelling and dry weight PEC and T-PEC3100 [24].

2.6. Microspheres (MS) preparation

Synthesis of MS was done by w/o emulsion solvent evaporation technique by using various combinations of PEC and optimized T-PEC3100 as this method is convenient and produce efficient results. Dispersed phase was prepared by dissolving Pec, T-PEC3100 and 1 % w/v LS (We prepared 1 mg/mL solution of LS for loading into the microspheres. LS were loaded during the process as this is simple process and offers uniform drug distribution into microspheres) in ratios 1:0:1, 1:1:1, 2:1:1 and 3:1:1. While the continuous phase was 250 mL liquid paraffin containing span-80 as an emulsifying agent. The dispersed phase was then emulsified in continuous phase in the form of tiny droplets. The principle behind emulsion formation is to achieve a stable dispersion of water droplets within the oil phase. This allows for efficient encapsulation of the drug within the microparticles. The resultant emulsion was then stirred at 500 rpm at 50 °C for 4 h. Rotary evaporator (Thermo-Scientific, RE2010) was used for the evaporation of the aqueous phase at 100.2 ± 0.5 °C. As, to separate out the microspheres the aqueous phase should evaporate at water/air interface. The aqueous phase evaporated, the microspheres hardened and started flowing freely. After the complete removal of water, liquid paraffin was decanted by washing MS several times with n-hexane, MS were collected on a Whatman's filter paper no. 40 and dried in an oven at 30 °C for 24 h [25].

2.7. Physicochemical characterizations and ex-vivo mucoadhesion

Physical characterization of MS including % Hydration, Hausner's ratio, Car's index, angle of repose, and average particle size (μm) of MS formulation i.e., MS1:0, MS1:1, MS2:1, and MS3:1 was performed by using already reported method by Al-Hashemi et., al [26]. Morphology and surface texture of MS1:1 LS loaded and without drug were observed by using SEM (Hitachi High Tech S4800 FE-SEM) after gold coating at accelerating voltage 5 kV Elucidation.*Ex-vivo* mucoadhesion study was conducted for MS formulation following the above, mentioned procedure by using modified USP I disintegration apparatus.

2.8. Encapsulation Efficiency(EE) and in-vitro release of LS

Actual drug loading in MS was determined by spectrometric analysis. 20 mg MS were crushed dissolved in 4 mL PBS and kept under stirring overnight. The suspension was then centrifuged for 20 min at 15,000 rpm. The supernatant was filtered and analyzed using UV–Vis Spectrophotometer (PerkinElmer, USA) at 220 nm. After that, the following formula was used for % EE

$$EE\ (\%) = \frac{W_t - W_f}{W_t} \times 100 \quad (2)$$

where W_t is the total LS and W_f is the free drug.

In-vitro percentage release of LS in 50 mg was calculated by using a USP II dissolution apparatus. Already immersed cellulose dialyzing membrane (12–14 Kad) was used as a diffusion barrier. An accurately weighed amount of all formulations containing 10 mg of LS were taken in dialyzing membrane with 2 ml PBS and immersed in 900 ml of 100 mM PBS at pH 6.8. The apparatus was operated at 100 rpm at 37 ± 0.5 °C. An aliquot of 5 mL was removed at predetermined time intervals at 0.5, 1, 2, 4, 6, 8, 10, and 12 h and replaced with the fresh buffer. The sample was analyzed for its drug content by measuring absorbance at 220 nm and the concentration of LS was calculated by using an already prepared standard curve of LS at 220 nm [27]. The release pattern was further justified by model-dependent approaches after applying zero and first-order, Higuchi's and Korsmeyer-Peppas's model according to the Akaike information criterion.

2.9. Trans well permeation studies

Intestinal mucous was collected using a slightly modified, previously reported method by Friedl et al., [28]. Briefly, the rabbit's intestinal mucous was mixed 0.1 M NaCl in a ratio of 1:5 and stirred for 2 h under controlled conditions of temperature −40 °C. For the removal of debris, mucous was centrifuged at 1000 rpm for 2 h, the clean bottom layer was collected and stored in the refrigerator at

−20 °C for further use. Permeation studies were performed by using Corning-TEK 24-well plate. Briefly, 50 μL of mucous was added to the trans-well and the acceptor chamber was filled with 500 μL with 100 mM phosphate buffer pH 6.8. 250 μL suspension of each formulation i.e., MS1:0, MS1:1, MS2:1, and MS3:1 was placed over the mucous membrane and the permeation was observed after continuous shaking over the orbital shaker (Thomas Scientific). 100 μL of the samples were drawn after predetermined time intervals i.e., 1, 2, 4, 6, and 8 h, respectively. The permeation percentage of LS was calculated at 220 nm by using an already prepared increasing concentration of LS. The apparent permeability and permeability coefficient was measured by using the following formulae:

$$P_{app} = \frac{Q}{C \times A \times T} \tag{3}$$

where P_{app} is the apparent permeability in cm/s, Q is the amount of molecule transported per min (μg/min), A is the area exposed to the mucous membrane in cm^2, C is the initial drug concentration of the drug in μg/mL [29]. Permeability results were reported as a mean of the standard deviation of a minimum of three autonomous experiments.

Solubility together with dose is important to estimate the maximum absorbable dose of a drug (MAD). Johnson and Swindell et., al proposed a simple method to determine MAD and fraction of drug absorbed of loxoprofen through the stomach.

$$MAD = s \times K_a \times SV \times STT \tag{4}$$

$$f_{(abs)} = \frac{MAD}{Dose} \tag{5}$$

where 's' is the solubility of the drug, 'K_a' is the absorption rate constant (min^{-1}) from rat intestinal perfusion test (simulated with human GIT), 'SV' is the volume i.e., 900 ml and, 'STT' is the transit time of mucoadhesive dosage form in GIT. $\mathbf{f_{(abs)}}$ is the fraction of drug absorbed.

2.10. Evaluation of anti-inflammatory activity and inflammatory markers

The anti-inflammatory effect of optimized MS1:1 was assessed by the rat paw edema inhibition method. Before the study, the protocol was approved by Research and Ethics committee, Faculty of Pharmacy, Bahauddin Zakariya University, Multan with the letter number EC.Fop786/12/2022. Briefly, adult Rats (subspecies: Rattus norvegicus Domestica) were taken of either sex with an average weight of 170 ± 10 gm and divided into 3 groups (n = 6) per Federation of European Laboratory Animal Science Association (FELASA) animal Guidelines and recommendations [30]. Rats were kept fasted overnight with adequate water before the activity. Negative Control Group (CR) given no treatment, Positive Control Group (ST) given 1 mg/mL solution of LS (on the basis of weight), and Group 3: MS treated rats (MT) given MS1:1 containing LS based on rats' weight. LS are already a proven drug for the treatment of mild to moderate pain and arthritis. The therapeutic dose is 60 mg BID. In this study, we calculated the dose of LS on the basis of Rats' individual weight by applying the following formula

$$\mathrm{Dr} = \frac{\mathrm{Dr} \times \mathrm{Da}}{\mathrm{St.\ Conc}} \tag{6}$$

where Dr is required dose for rats, Da is the actual dose of the drug from previous literature. After 30 min, the right hind paw was injected subcutaneously with 0.1 mL of 1 % carrageenan solution as shown in the graphical abstract. The volume of the rat paw was measured with a plethysmometer immediately before and after the administration of carrageenan. Rat paw volume was measured at 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h, respectively. The percentage inhibition was recorded and compared to the positive control [31].

$$Edema\ Inhibition\ (EI)\ \% = \frac{EIc - EIt}{EIc} * 100 \tag{7}$$

where EIc is edema inhibition n control group and EIt is edema inhibition in treated group.

In each experimental group 6 animals were kept ($n = 6$). Serum samples of all animals were collected and each serum samples was tested in triplicate and statistically analyze the findings of each group. After completion of mice paw edema study, animals were sacrificed under anesthesia by cervical dislocation. Fresh blood samples were collected by cardiac puncture and centrifuged at 6000 rpm to collect serum. The white upper layer of blood serum was collected and stored at −80 °C. In EISA test, 100 μL of each sample was added and further incubated according to kit manufacturer's protocol. Serum samples were isolated and the level of inflammatory cytokines were estimated according to manufacturer's protocol by using commercial kit (Wuhan Zokeyo Biotechnology Co, Ltd. (Rat TNF-α Elisa kit, Catalogue # Y-83079-48T) and (Rat IL-6 Elisa kit, Catalogue # Y-84561-48T).

2.11. Statistical analysis

The data were analyzed by one-way analysis of variance ANOVA to compare the mean of all values to calculate the level of significance, $p < 0.05$, mean and standard error of the mean SEM < 1.5. ANOVA was done by using Microsoft Excel 2013 and IBM SPSS Statistics 25.

3. Results

Low methoxy PEC was modified to thiolated pectin through esterification reaction using thioglycolic acid as thiol (-SH) donating reagent. The reaction took place for 3 h at 80 °C. Purification of T-PEC was confirmed by the removal of unbound thiol groups by using a dialysis membrane and the product was then grounded to make off-white powder with maximum % yield i.e., 78.12 ± 2.5 %.

3.1. Characterization of T-PEC

3.1.1. Thiol group content and charring point determination

The effect of the increasing concentration of TGA was observed on the thiolation of PEC and results are displayed in Table 1. In this study, we studied the introduction of thiol group up to 5X of thioglycolic acid concentration. Results show that the quantity of thiol group immobilized on PEC increased with the increase in the TGA concentration. Maximum thiol content was determined i.e., 3100 ± 0.125 μmol/gm, and this formulation were then used in microsphere synthesis.

The Charring point of PEC and T-PEC3100 was 201 ± 0.5 °C and 174.14 ± 0.5 °C respectively. And all other formulations of T-PEC also showed a decrease in charring point which also confirms the structural changes in PEC. Substitution of the hydroxyl group in PEC with thiol was confirmed by Elman's colorimetric assay is shown in Table 1.

3.1.2. SEM, FTIR, XRD & DSC

The PEC showed smooth and shinny surface as shown in Fig. 2(A&C) and T-PEC3100 shows scaly, rough, and porous surface as mentioned in Fig. 2(B&D). In Fig. 3 (A), the FTIR spectrum of PEC exhibited a distinct band of –OH stretching of alcohol at 3297 cm^{-1}. The band that appears at 1735.38 cm^{-1} can be assigned to C=O stretching of the esterified carbonyl group. Other peaks are C–H bending at 1222 cm^{-1} and C–O stretch of primary alcohol at 1007 cm^{-1}. In the spectrum of T-PEC3100, there's an additional distinct peak at 2820 cm^{-1} which confirms the presence of –SH group. Other bands include –OH stretch at 3352 cm^{-1}, C=O stretch of ester at 1720 cm^{-1}, the stretch of –COOH at 1219 cm^{-1}, and H–C–H wagging at 1150 cm^{-1}. DSC results were shown in Fig. 3(B). The thermogram of PEC showed a broad endotherm at 80 °C and 210 °C with the heat of fusion 501.51 J/g, while T-PEC3100 thermogram showed a decrease in endothermic transition temperature at 100 °C, 255 °C, and 320 °C with the heat of fusion 452.52 J/g which confirms the more stability of T-PEC3100. In Fig. 3(C), the results of the X-ray diffraction spectra of both PEC and T-PEC3100 were compared. X-rat diffractogram of PEC showed peaks at 10° and 15° (2θ) while in the diffractogram of T-PEC3100 peaks appeared at 13° and 22° (2θ). The peak intensity of T-PEC3100 is greater than PEC which also indicates comparative crystalline nature of T-PEC3100 [32]. PEC is slightly crystalline in nature depending upon the course of its extraction.

3.2. Ex-vivo mucoadhesion and swelling evaluation

The swelling behavior of PEC and T-PEC3100 is shown in Fig. 3(D). The effect of the buffer's pH on the swelling of PEC and T-PEC3100 was compared up to 5 h and small surface erosion was observed in the case of PEC which was absent in T-PEC3100 and showed its stability in both pH 1.2 and 6.8. Equilibrium swelling of T-PEC3100 was exhibited after 4 h and showed no erosion ($p < 0.01$), which may be due to their improved cohesive strength after thiolation. Moreover, 2.1 and 3.5 fold ($p < 0.05$) increase was observed in the initial weight of T-PEC3100 at pH 1.2 and 6.8 respectively. The mucoadhesion characteristics of T-PEC were found to be associated with its thiol content as shown in Table 1. T-PEC3100 disc remained adhere with the membrane for more than 2 h while the disc of PEC got detached after 40 min. T-PEC3100 contains the highest amount of thiol groups present per mole of the PEC and exhibited 3 folds increased mucoadhesion time and p value was less than 0.01 as mentioned in Table 1. All formulations of T-PEC exhibited higher swelling as compared to original pectin. The hydration theory of mucoadhesion supports that higher swelling leads to more mucoadhesion characteristics of biodegradable polymers. Similarly, T-PEC3100 exhibited higher swelling rate which ultimately led to more mucoadhesive characteristics. The discs of T-PEC remained attached to rabbit mucosal surface for more than 2 h.

3.3. Microspheres synthesis

Different formulations of LS-loaded MS with varying concentrations of PEC: T-PEC3100 (1:0, 1:1, 2:1, 3:1) were synthesized using

Table 1
Comparison of thiol content (μmol/gm), charring point and, mucoadhesion time (h) of T-PEC formulations.

Formulations of T-PEC	PEC: TGA	Thiol content (μmol/gm)	Charring point (°C)		Mucoadhesion time (h)
			TGA	96.5 °C±0.5	
			PEC	201.3 °C±0.5	
T-PEC1080	1:1	1080 ± 1.3	198.2 ± 0.5 °C		0.9 ± 0.02*
T-PEC1290	1:2	1290 ± 1.2	194.1 ± 0.5 °C		1.3 ± 0.02**
T-PEC1570	1:3	1570 ± 0.5	195.3 ± 0.5 °C		1.6 ± 0.05*
T-PEC1860	1:4	1860 ± 1.4	196.2 ± 0.5 °C		1.9 ± 0.03*
T-PEC3100	1:5	3100 ± 1.5	174.4 ± 0.5 °C		2.1 ± 0.03**

p value *<0.05 and **<0.01.

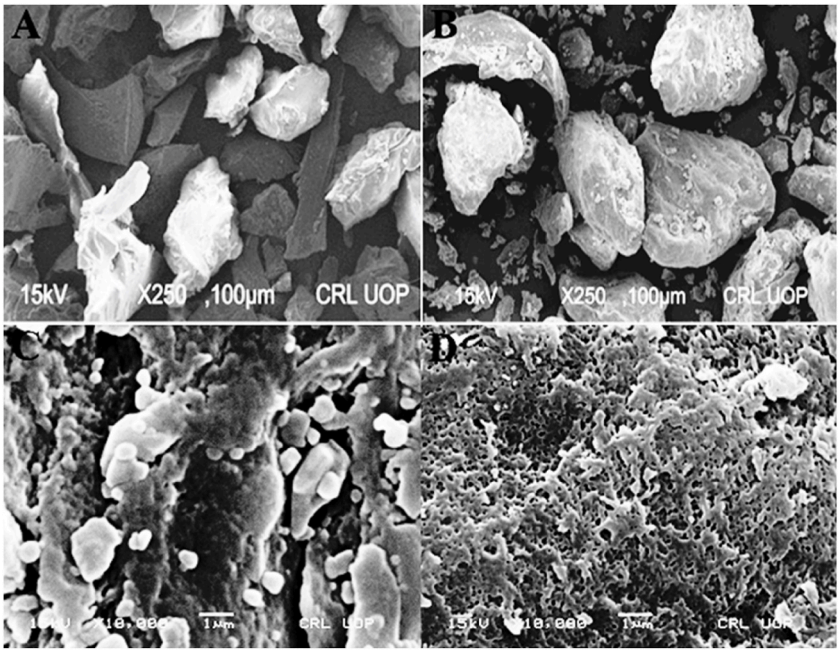

Fig. 2. SEM images of pectin (A&C) and optimized T-PEC3100 (B&D).

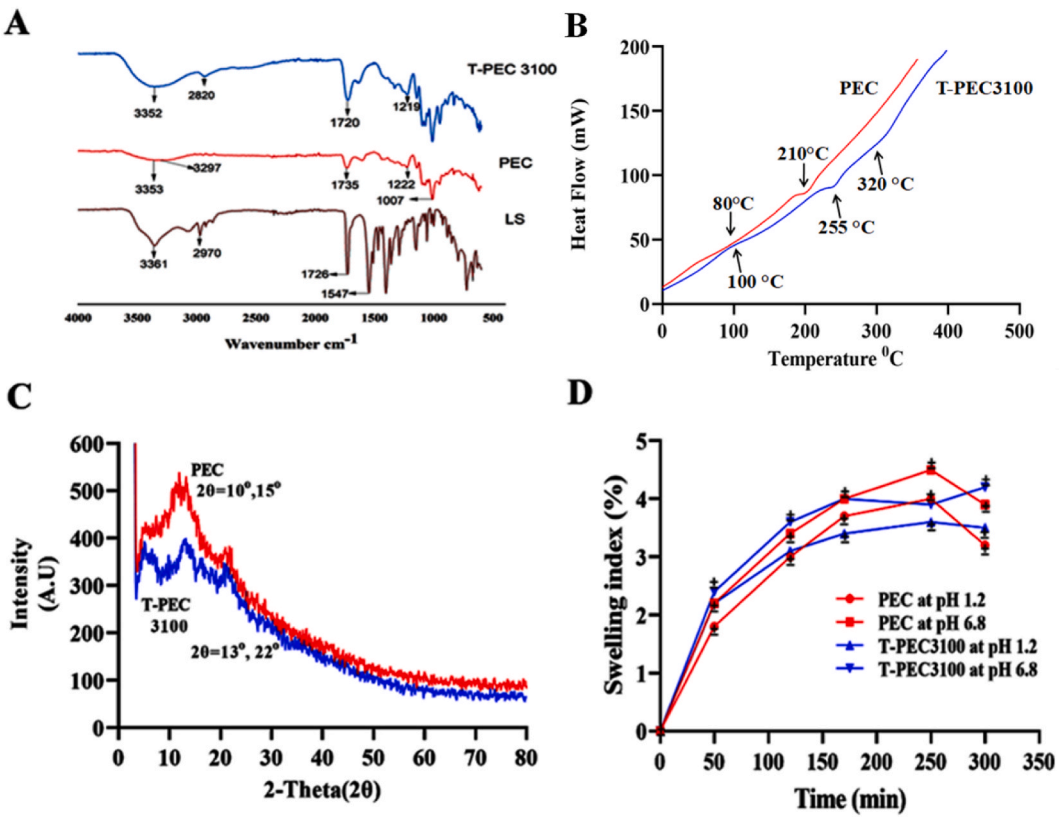

Fig. 3. FTIR spectra of PEC, loxoprofen and T-PEC3100 (A), DSC curves of PEC and T-PEC3100 (B), XRD images of PEC and T-PEC3100, (C) percentage swelling index of PEC and T-PEC3100 in pH 1.2 and 6.8 buffer media (D).

the w/o solvent emulsion evaporation technique. All MS formulations showed an average particle size of less than 10 μm as illustrated in Table 2.

Hausner's ratio and car's index of all formulations were less than 1.25 and 16 % respectively, indicating good flow of MS. The angle of repose of all formulations was greater than 30° again indicating freely flowing powder as shown in Table 2.

3.4. Physicochemical characterizations

SEM images confirmed the formation of MS in the size range of 1–10 μm. Fig. 4 (A&C) showed MS1:1 (LS unloaded) with a smooth surface. The coarse surface in part B&D confirmed the loading of LS in MS 1:1 as shown in Fig. 4 (B&D). These micron-sized particles can be used for local and targeted drug delivery. As shown in SEM images, LS was evenly distributed amongst these MS and there was no burst release in in-vitro analysis. Table 2 displays the results of % hydration of MS formulation and T-PEC3100 was observed to have a 1.5-fold increase in % hydration. Disc adhesion method confirmed the adhesion time of MS1:0 (contained only PEC) and showed the least mucoadhesion up to 1.89 ± 0.256 h while all formulations containing T-PEC displayed increasing mucoadhesive time i.e., MS1:1, MS2:1and MS3:1 displayed 4.2 ± 0.125, 2.6 ± 0.425 and 2.11 ± 0.125 h respectively, in Fig. 5(A). MS1:1 had an equal concentration of PEC and T-PEC3100 remained adhered to the mucosal surface for a longer period even after 4.3 h.

3.5. Encapsulation efficiency (EE) and % drug release

The percentage encapsulation efficiency (EE%) of all formulations is given in Table 2. MS1:1 showed the highest % encapsulation efficiency of LS while MS1:0 showed the least concentration which may be attributed to the thiolation of pectin. As the drug loading was entrapped inside the microspheres, so the burst release of drug is not very significant. Moreover, Loxoprofen sodium has far less mucosal irritant effects as compared to other NSAIDs. But, in case of arthritis as the drug has to be used on regular basis, it can cause gastric and intestinal irritation. That's why the initial burst release would not cause much harm. In Fig. 5(B) *in-vitro*, drug release studies at 100 mM phosphate buffer (PBS) pH, 6.8 were shown. Percentage release was calculated for each formulation at different time intervals up to 12 h. As the results indicated that formulations MS1:0, MS1:1, MS2:1, and MS3:1 showed 87.25, 75.75, 82.51, and 89.43 % ($p = 0.01$) drug release for 12 h. The sustained release microspheres prevented the burst release of LS, releasing the drug slowly in a prolong manner. As a result, the drug is available for longer time but in less amount which avoids the mucosal irritating effects of Loxoprofen. In Table 3, various release models i.e., zero-order, first-order, Higuchi's model, and Korsmeyer-Peppas model were applied to study the release pattern of LS.

The values of R^2 in first-order release models were near to one, as compared to other models, which showed that % drug release is following first-order release mechanism. Akaike information criterion (AIK) was used to select the optimized release MS1:1 i.e., 30.875. Further n value of Korsmeyer-Peppas model was observed for the study of Fickian/non-Fickian evaluation. If the value of 'n' lies within 0.4–0.8 it indicates non-fickian. So, the LS release results showed that values of 'n' were in the range and hence the formulations exhibited non-fickian flow.

3.6. Ex-vivo permeation study

The cumulative percentage permeation was calculated and shown in Fig. 5(C). The various formulations i.e., MS1:0, MS1:1, MS2:1, and MS3:1 have 75.15, 85.25, 70.65, and 72.25. LS standard was used as positive control and the well without mucous membrane was used as a negative control. LS permeation was 95.55 % ($p = 0.001$) but only for 3h and remained constant. The apparent permeability of all MS formulations was calculated at 12.9–32.5 cm/s ($p = 0.01$) and maximum permeability was calculated in the case of MS1:1 due to an equal ratio of PEC and T-PEC3100. MAD and the fraction of drug absorbed through MS1:1 through the mucous membrane were calculated i.e., 400 μg and 0.85 respectively as shown in Fig. 5(D). The permeation results were further evaluated by post hoc test which showed the p value was less than 0.001 indicated the results are statistically significant.

3.7. Evaluation of anti-inflammatory activity and inflammatory markers

Fig. 6(A) illustrated the mean % inhibition of edema in negative control, Loxoprofen treated group and microspheres treated groups. The % inhibition of edema was 1.5 folds in MT group as compared to the ST. The group ST showed edema inhibition as early as 0.5 h, but the effect remained the same for 3 h. Later, in the ST group, inhibition was less as compared to MT treated group because of the short elimination half-life of LS i.e., 75 min. MS1:1 formulation in MT initially showed a rapid reduction in edema because of the presence of LS in the free form at the surface of MS and can be attributed to the synergistic anti-inflammatory effect of PEC itself [33].

Table 2
Micromeritics properties, particle size (μm), and entrapment efficiency (%) of MS formulations.

MS	PEC: T-PEC3100	Hausner's ratio	Car's index	Angle of repose	Particle Size (μm)	Entrapment efficiency (%)
MS1:0	1:0	1.16 ± 0.02	12.83 ± 0.35	22.34 ± 0.05	2.6 ± 0.40	49.25 ± 0.125
MS1:1	1:1	1.19 ± 0.02	14.99 ± 0.02	26.16 ± 0.02	6.0 ± 0.63	64.65 ± 0.025
MS2:1	2:1	1.2 ± 0.02	16.01 ± 0.02	25.36 ± 0.5	4.7 ± 0.57	54.25 ± 0.124
MS3:1	3:1	1.22 ± 0.02	15.99 ± 0.35	28.71 ± 0.2	9.0 ± 0.77	60.36 ± 0.134

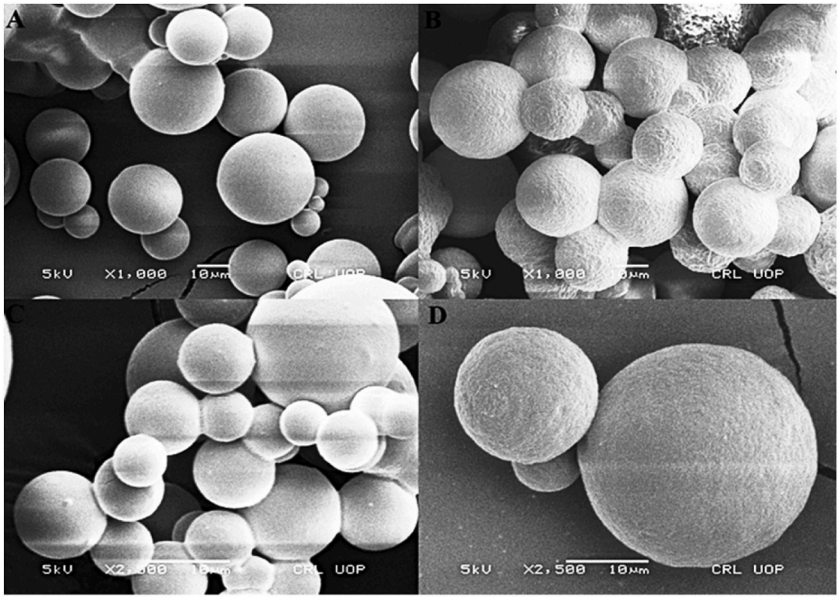

Fig. 4. SEM photomicrographs of (A&C) MS1:1 without the drug having a smooth and shiny surface, (B&D) MS1:1 drug-loaded are rougher.

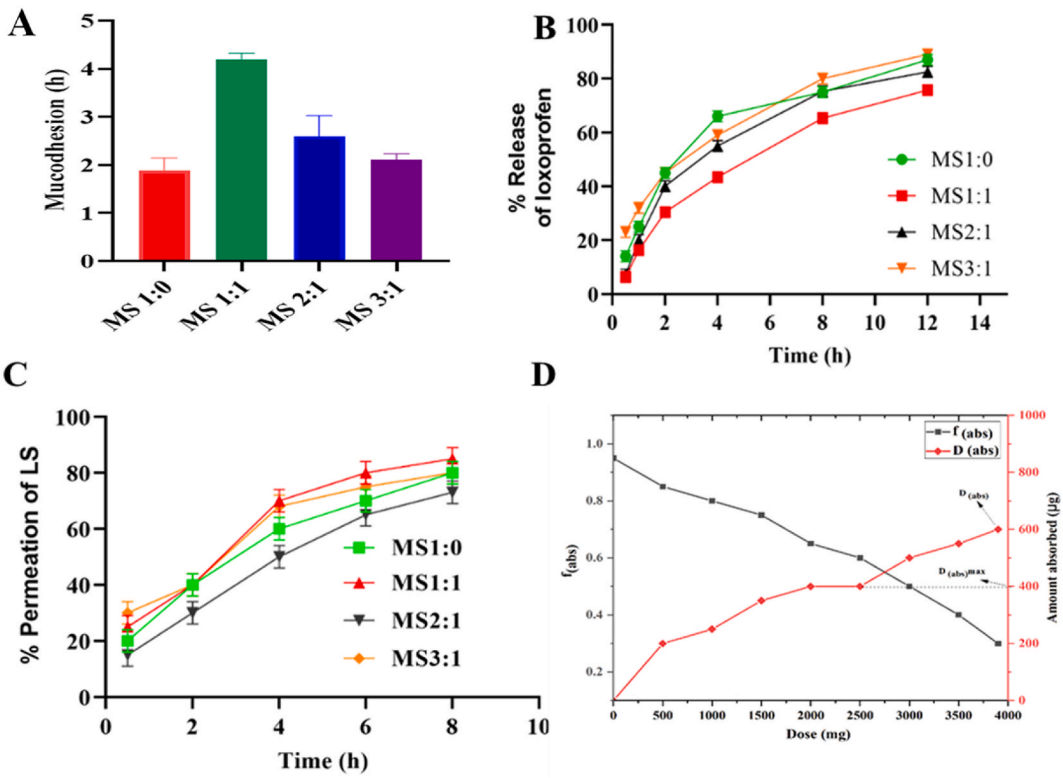

Fig. 5. (A) Mucoadhesive time of MS formulations (B) Percentage release of LS from MS, (C) Cumulative percentage permeation of LS from MS formulations across the mucous, (D) Maximum absorbable dose and the fraction of drug absorbed.

The serum level of pro-inflammatory cytokines TNF-α and IL-6 was significantly raised in inflammation model group compared to control group. However, treatment with MS formulation markedly decreased the raised level of inflammatory cytokines similar to lexoprofen sodium treatment group as shown in Fig. 6(B and C). Fig. 7(A–D) shows the inhibition of edema in microspheres treated group (RM) at different time intervals (0–48 h), showing how the edema was decreased over time. The statistical analysis was further

Table 3
Model-dependent approaches for in-vitro drug release parameters (K0 = rate constant, R2 = regression model, AIC = Akaike Information Criterion) of Loxoprofen from different formulations of microspheres (MS).

Formulation	Zero-order kinetics			First-order kinetics			Higuchi's model			Korsmeyer-Peppas model		
	K_0 (h^{-1})	R^2	AIC	K_1 (h^{-1})	R^2	AIC	K_H (h^1)	R^2	AIC	n	R^2	AIC
MS1:0	8.855	0.4178	12.097	0.244	0.9418	25.523	27.056	0.9305	17.1698	0.448	0.9552	32.097
MS1:1	7.289	0.8071	20.566	0.135	0.9978	30.875	21.539	0.9640	22.767	0.592	0.9798	35.875
MS2:1	8.321	0.6513	27.980	0.191	0.9662	20.576	25.008	0.9434	25.082	0.521	0.9504	24.984
MS3:1	9.062	0.2598	35.875	0.256	0.9984	19.451	27.799	0.9609	29.986	0.410	0.9946	19.875

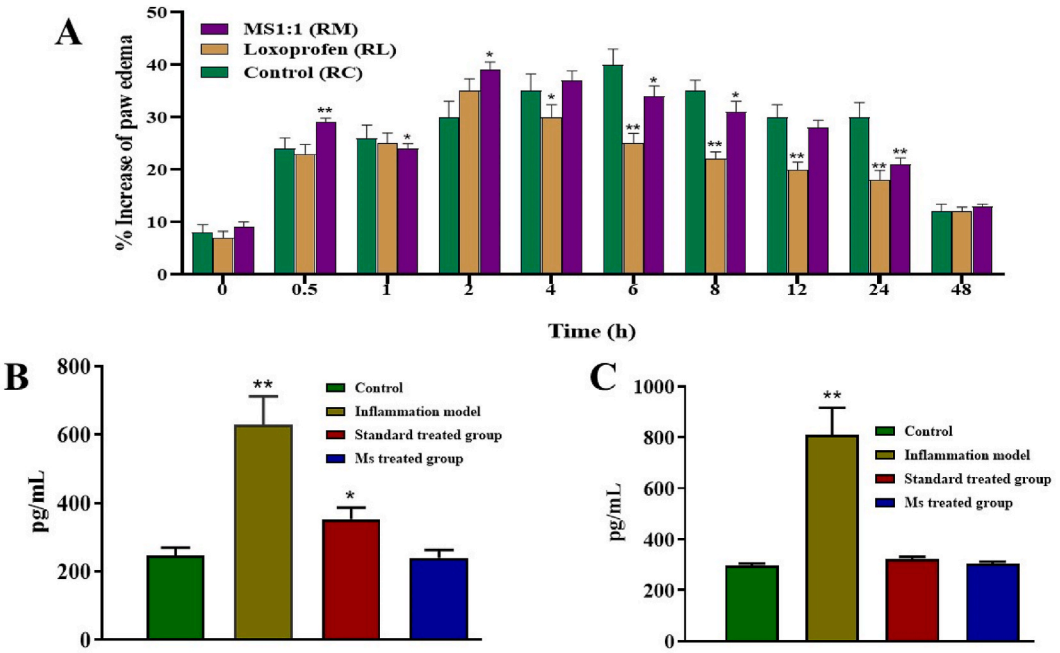

Fig. 6. Percentage inhibition of edema in negative control group (RC), Loxoprofen treated (Positive control group) (RL) and loxoprofen loaded MS1:1 treated group (RM) (A), infalmmatory markers TNF-α (B) and IL-6 (C). In Fig. 6A and B and C (***P < 0.001, **P < 0.01, *P < 0.05).

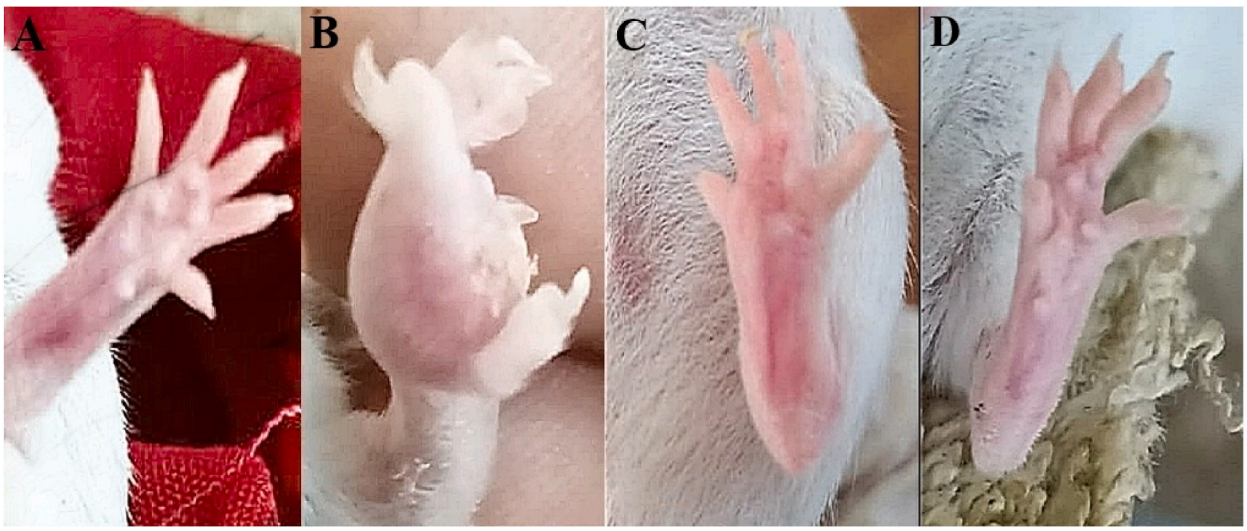

Fig. 7. Visual representation of rat paw before and after edema induction at time (A) 0 h, (B) 1 h, (C) 12 h, (D) 48 h.

evaluated by post hoc test which showed the p value was less than 0.0001 indicated the results are statistically significant.

4. Discussion

Low methoxy PEC was conjugated with TGA through a strong covalent bond in an esterification reaction. Theoretically, TGA can be linked with any hydroxyl group in PEC, but the primary alcohol might be more preferred site for substitution. During thiolation of pectin, increasing the quantity of thioglycolic acid led to higher amount of free available thiol groups. The phenomenon can be attributed to stoichiometry of this esterification reaction, which involves the replacement of hydroxyl groups with thiol (-SH) available in pectin's backbone resulting in an ester bond. Now, with the increase in molar concentration of TGA, increased the number of thiol group available for participating in the reaction. This led to higher number of esterification reactions occurring, and caused a greater number of free thiol group incorporation into pectin's molecule. T-PEC3100 was considered an optimized formulation for synthesizing MS formulation because of its high *ex-vivo* mucoadhesion duration which was three folds increased as compared to PEC [33]. Thiolation of low methoxy PEC was considered more effective as compared to previously reported T-PEC having a % yield of 48.12 ± 0.12 by R. Sharma et al. which was likely, due to the presence of more carboxylic groups in low methoxy PEC [14].

SEM results displayed the comparable fibrous like the structure of T-PEC which was also reported by Chewatana kornkool et al., [34]. The surface morphology of both PEC and T-PEC3100 was studied using SEM at LPM and HMP displayed mode [35]. According to the results of Elman's assay, increased thiol content from T-PEC1080 to T-PEC3100 was possible due to the increased molar concentration of TGA used in the reaction. The concentration of TGA was studied as an independent factor to evaluate its effect on thiol group quantity and ultimately on how well the mucoadhesion of PEC was improved. The compartive thiols contents and percent drug release from previously developed microparticles with the developed formulation are shown in Table 4. The results went well with the hypothesis and the thiol-rich formulation displayed the highest mucoadhesion duration. Sharma et., al, also reported a similar behavior of concentration dependency of thiol content on mucoadhesion time using thiourea instead of TGA [14]. After selecting the optimized formulation, T-PEC3100, it was subjected to further analytical and physicochemical characterization.

Reduction in the charring point of T-PEC was observed which confirms the modification and is attributed to the presence of –SH. Similar results were reported by Satheeshababu et., al by determining the reduction in the charging point of thiolated and non-thiolated chitosan from 258 ± 1.2 °C to 190 ± 1.2 °C [40]. FTIR technique is a powerful tool for the characterization of organic molecules, considering that it is sensitive to functional groups such as esters, hydroxyl, carbonyl, amides, thiol etc. In FTIR spectra the presence of an additional –SH group stretch peak confirmed the presence of the thiol group in the T-PEC3100 which was absent in the spectrum of PEC [41].

The presence of extra peaks at different temperatures in DSC thermogram further confirmed the modification of the introduction of thiol moieties in T-PEC which were absent in original PEC. XRD results, also clearly showed a difference in the crystallinity of both polymers. T-PEC3100 after thiolation showed three distinct sharp peaks confirming its crystalline nature as compared to the PEC. Similar results are already reported by Sharma et al., [14]. Equilibrium swelling of T-PEC3100 was exhibited after 4 h and showed no erosion which is likely to be attributed to their improved cohesive strength after thiolation. Bernkop et al. reported that thiolation increases the water-absorbing capacity of polymers. The water uptake capacity of the polymer has a great influence on their mucoadhesive property as on absorbing water from mucosal surface polymeric chains intermingle with the mucous membrane and adhere more strongly [42]. Although the higher swelling resulted in mucilage formation on the surface which was observed in the case of PEC, but T-PEC3100 did not show such a slimy surface. The reason for this higher swelling was the activation of carboxyl groups of polymers at higher pH.

Swelling properties of T-PEC are linked with the mucoadhesion studies as the water uptake ability of thiolated conjugate enhances the durable contact with the mucus membrane. A rise in mucoadhesion time was observed in all formulations of T-PEC with the highest exhibited by T-PEC3100. Thiol group interacts with the cystine present in the mucosal glycoproteins and form strong disulfide linkages (-S-S-) which allows the thiolated polymers in this case T-PEC, to stay on the mucous membrane for longer. While PEC, is itself a mucoadhesive polymer because of the presence of carboxylic groups which make ionic interactions with the mucin but due to lack of the thiol moiety couldn't stay on the mucosal surface foe extended time. Similar results are already reported in many studies where many natural and synthetic polymers have exhibited prolong mucoadhesive time. Sodium alginate, Hyaluronic acid, Moringa gum and, Chitosan have shown 2, 6.5, 1.5 and 2.48 folds increase in mucoadhesive strength respectively after thiolation [43].

Results of particle size of MS showed an increase with an increase in polymer load from MS1:0 to MS3:1, which may be due to increased intermolecular repulsion [44]. The possible reason for the increased % hydration of all formulations was that both polymers were hydrophilic and absorbed enough amount of water. So as the polymer load was increased, the amount of retained water in the microsphere was also increased thus increasing the % hydration [45]. The coarse surface in Fig. 5(B&D) confirmed the loading of LS in MS1:1. These micron-sized particles can be used for local and targeted drug delivery. As shown in SEM images, LS was evenly distributed amongst these MS and there was no burst release in *in-vitro* analysis.

The combination of PEC and T-PEC3100 in MS formulations overcomes the stability problem of PEC containing MS by forming strong intermolecular attraction. Maximum EE (64.65 ± 0.025 %) of MS1:1 confirmed the availability of more area for loading for LS as compared to MS1:0, MS1:2, and MS1:3. Similar results are also explained in our previous study by thiolated sodium alginate MS with entrapment efficiency of 44.54 ± 1.25 %. A similar trend of mucoadhesion duration was observed in MS formulation, in which MS1:1 formulation showed the highest mucoadhesion time compared to the other three formulations, with MS 1:0 (containing PEC only) showing the least. Comparing the drug release from all formulations it was observed that T-PEC3100-rich formulation (MS1:1) didn't show a burst release of LS. As the concentration of T-PEC3100 decreased in other formulations it caused high drug release initially, which also explains that thiolation improved the cohesiveness of the polymer so, the drug is more efficiently entrapped. Due

Table 4
Comparative analysis of thiolated pectin with already reported data of anionic polymers.

Polymers	Alginates	Hyaluronic acid	Gelatin	Pectin	Pectin in this study
Thiol content	320–730 μmoL/g	201.3 μmoL/g	362 μmoL/g	892.27 ± 68.68 mmol	1030-3100 μmoL/g
Study design	*In-vitro*/microspheres	*In-vitro*/tablets		*In-vitro*/Microparticles	*In-vitro-In-vivo*/Microspheres
Mucoadhesion	3X	6.5X	4X	5X	3X
Permeation	–	–	–	1.6X	Increased
Drug release	Controlled drug release (>80 %)	Sustained drug release	–	Not considerable	>75 %
References	36	37	38	39	

to the formation of a disulfide network in the polymer, drug molecules diffuse at a slower rate and less release was observed. Similar behavior was already reported by Cheewatana, K. et al., [46]. Moreover, MS1:1 was found one of the promising candidates for providing SR of LS because of the higher surface-to-volume ratio available.

The sustained release of Loxoprofen from mucoadhesive microspheres, allows the drug to be available for gradual and prolong contact. This helps to reduce the drug concentration at a given time, which minimize the cyclooxygenase (COX) inhibition and succeeding reduction in prostaglandins concentration. In the results, the risk of mucosal damage is minimized. LS containing matrix tablets and multiparticulate systems are proved to provide drug release in sustained manner for prolong duration of time which enhances patient compliance and decrease incidence of side effects related to multiple, prolong dosing of LS.

We assessed the relation of different parameter on the absorbing dose such as its solubility, transit time of dosage form and volume of fluid in intestinal region. As the dosage forms were mucoadhesive, their transit time was increased and the MAD was also increased. Basically, the MAD is the amount of drug absorbed till the absorption limit is reached. But, in practice that limit is not achieved, so we compare permeation of asset of compounds. In our study we compared the permeation through the microspheres composed of only PEC and then with the combination of PEC & T-PEC. Anionic polymers have already been reported to improved permeation after thiolation which is likely to be the result of interaction of thiol group with cystine rich subdomains of mucous and developing disulfide linkages, enhancing the permeation of material. Permeation results are also justified by previous studies. Similar behavior is recently reported in another study where three generations of T-PEC are compared. PEC alone showed very less mucous permeation as compared to thiolated and S-protected T-PEC.

The slow inhibition of inflammation even after 24 h was observed in MT due to SR behavior of MS as compared to CR group. The MS1:1 treated group showed promising effects as edema was significantly inhibited for 24 h making it a good choice for an SR delivery system. Moreover, results also justify the mucoadhesive properties of MS. Perera et al. have also reported that T-PEC microparticles disintegrate slowly which releases and provide the drugs in the gastrointestinal system for a longer period. The sustained anti-inflammatory effect was attributed to the T-PEC. This sustained anti-inflammatory effect was attributed to the presence of T-PEC in the formulation as it captured the drug within the polymeric network and let it release slowly.

5. Conclusions

Thiolation of PEC by using different ratios of thioglycolic acid was processed and one optimized formulation (T-PEC3100) based on the thiol content was selected for MS synthesis and in-vivo evaluation. It was found that amount of thiol groups immobilized on polymer and the duration of mucoadhesion were dependent upon the amount of TGA. The investigations concluded that results from the *in-vitro* characterizations, mucous permeation, and *in-vivo* SR effect of LS from MS formulations, evidenced the thiolation as a promising approach to increase the mucosal residence time of formulations. Moreover, mucoadhesive MS can be used as an approach to provide sustained drug release to counter the mucous membrane lesions caused by LS.

Ethics statement

The protocol for anumal study was approved by Research and Ethics committee, Faculty of Pharmacy, Bahauddin Zakariya University, Multan with the letter number EC.Fop786/12/2022.

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CRediT authorship contribution statement

Aisha Anam: Writing – original draft, Methodology. **Ghulam Abbas:** Writing – original draft, Software, Conceptualization. **Shahid Shah:** Writing – review & editing, Investigation, Data curation. **Malik Saadullah:** Writing – original draft, Funding acquisition, Formal analysis. **Dure Shahwar:** Writing – original draft, Methodology, Investigation. **Khalid Mahmood:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Muhammad Hanif:** Writing – original draft, Supervision, Project

administration, Conceptualization. **Nabeel Ahmad:** Writing – original draft, Visualization, Validation. **Ejaz Basheer:** Writing – original draft, Methodology, Data curation. **Ahmad J. Obaidullah:** Writing – review & editing, Resources, Funding acquisition. **Hadil Faris Alotaibi:** Writing – review & editing, Resources, Funding acquisition. **Mohammed Alqarni:** Writing – review & editing, Resources, Funding acquisition. **Nabeela Ameer:** Writing – review & editing, Investigation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] I.B. McInnes, G. Schett, The pathogenesis of rheumatoid arthritis, N. Engl. J. Med. 365 (23) (2011) 2205–2219.
[2] J.E. Hyun, D.W. Li, E.B. Lee, C.S. Jeong, Anti-inflammatory, analgesic and antipyretic activities of loxoprofen sodium given intramuscularly in animals, Arch Pharm. Res. (Seoul) 24 (6) (2001) 541–545.
[3] S. Adepu, S. Ramakrishna, Controlled drug delivery systems: current status and future directions, Molecules 26 (19) (2021) 5905.
[4] (a) V. Grabovac, D. Guggi, A. Bernkop-Schnürch, Comparison of the mucoadhesive properties of various polymers, Adv. Drug Deliv. Rev. 57 (11) (2005) 1713–1723;
(b) N. Munot, U. Kandekar, C. Rikame, A. Patil, P. Sengupta, S. Urooj, A. Bilal, Improved mucoadhesion, permeation and in vitro anticancer potential of synthesized thiolated Acacia and karaya gum combination: a systematic study, Molecules 27 (20) (2022) 6829.
[5] (a) A. Bernkop-Schnürch, Thiomers: a new generation of mucoadhesive polymers, Adv. Drug Deliv. Rev. 57 (11) (2005) 1569–1582;
(b) C. Leichner, M. Jelkmann, A.J. A.d. d. r. Bernkop-Schnürch, Thiolated Polymers: Bioinspired Polymers Utilizing One of the Most Important Bridging Structures in Nature, 151, 2019, pp. 191–221.
[6] P.J.S.U.I.J. Sriamornsak, Chemistry of pectin and its pharmaceutical uses, A review 3 (1-2) (2003) 206–228.
[7] N. Thirawong, J. Nunthanid, S. Puttipipatkhachorn, P.J. E.j. o.P. Sriamornsak, Biopharmaceutics, Mucoadhesive properties of various pectins on gastrointestinal mucosa: an in vitro evaluation using texture analyzer 67 (1) (2007) 132–140.
[8] N. Thirawong, J. Thongborisute, H. Takeuchi, P.J. J.o. c. r. Sriamornsak, Improved intestinal absorption of calcitonin by mucoadhesive delivery of novel pectin–liposome nanocomplexes 125 (3) (2008) 236–245.
[9] Z. Özbaş, B. Özkahraman, Z.P. Akgüner, A. Bal-Öztürk, Evaluation of modified pectin/alginate buccal patches with enhanced mucoadhesive properties for drug release systems: in-vitro and ex-vivo study, J. Drug Deliv. Sci. Technol. 67 (2022) 102991.
[10] R. Sharma, M. Ahuja, H. Kaur, Thiolated pectin nanoparticles: preparation, characterization and ex vivo corneal permeation study, Carbohydrate Polymers 87 (2) (2012) 1606–1610.
[11] P. Venkatesan, R. Manavalan, K.J. J.o. b. Valliappan, c. pharmacy, Preparation and evaluation of sustained release loxoprofen loaded microspheres 2 (3) (2011) 159.
[12] X. Liu, X. Li, R. Zhang, L. Wang, Q.J.M.L. Feng, A Novel Dual Microsphere Based on Water-Soluble Thiolated Chitosan/mesoporous Calcium Carbonate for Controlled Dual Drug Delivery, 285, 2021 129142.
[13] R. Sharma, M. Ahuja, H. Kaur, Thiolated pectin nanoparticles: preparation, characterization and ex vivo corneal permeation study, Carbohydrate Polymers 87 (2012) 1606–1610.
[14] R. Kumar, T. Islam, M.J. Nurunnabi, Mucoadhesive carriers for oral drug delivery, J. Contr. Release 351 (2022) 504–559.
[15] N. Abbas, A. Rasul, G. Abbas, S. Shah, M. Hanif, Targeted delivery of aspirin and metformin to colorectal cancer using disulfide bridged nanoparticles of thiolated pectin and thiolated Eudragit RL100, Mater. Today Commun. 35 (2023) 105586.
[16] M.A. Anaya Castro, I. Alric, F. Brouillet, J. Peydecastaing, S.G. Fullana, V. Durrieu, Spray-dried succinylated soy protein microparticles for oral ibuprofen delivery, AAPS PharmSciTech 20 (2019) 1–10.
[17] C.E. Ugwu, F.C. Kenechukwu, E.O. Diovu, H.O. Udodeme, M.A. Momoh, E.B. Onuigbo, A.A. Attama, Exploitation of capsule system for colon targeted drug delivery of biopolymer-based microparticles: in vivo and in vitro applications, Heliyon 8 (2022) e00938.
[18] R. Sangsuwan, J.H. Yik, M. Owen, G.-Y. Liu, D.R. Haudenschild, J.S. Lewis, Intra-articular injection of flavopiridol-loaded microparticles for treatment of post-traumatic osteoarthritis, Acta Biomater. 149 (2022) 347–358.
[19] R.M. Dicharry, P. Ye, G. Saha, E. Waxman, A.D. Asandei, R.S. Parnas, Wheat Gluten− Thiolated poly (vinyl alcohol) blends with improved mechanical properties, Biomacromolecules 7 (10) (2006) 2837–2844.
[20] R. Sharma, M. Ahuja, Thiolated pectin: synthesis, characterization and evaluation as a mucoadhesive polymer, Carbohydrate Polymers 85 (3) (2011) 658–663.
[21] A. Bernkop-Schnürch, M. Hornof, T. Zoidl, Thiolated polymers—thiomers: synthesis and in vitro evaluation of chitosan–2-iminothiolane conjugates, International journal of pharmaceutics 260 (2) (2003) 229–237.
[22] K. Cheewatanakornkool, S. Niratisai, P. Sriamornsak, Bioadhesiveness of thiolated pectin for buccal delivery of carbenoxolone sodium, Asian J. Pharm. Sci. 11 (1) (2016) 124–125.
[23] S. Sonawane, M. Bhalekar, S. Shimpi, Preparation and evaluation of microspheres of xyloglucan and its thiolated xyloglucan derivative, Int. J. Biol. Macromol. 69 (2014) 499–505.
[24] M. Davidovich-Pinhas, O. Harari, H. Bianco-Peled, Evaluating the mucoadhesive properties of drug delivery systems based on hydrated thiolated alginate, J. Contr. Release 136 (1) (2009) 38–44.
[25] Jain, P.; Bhatt, D.; Jindal, D. K., RECENT ADVANCES IN MUCOADHESIVE MICROSPHERES BASED NOVEL DRUG DELIVERY SYSTEMS.
[26] H.M.B. Al-Hashemi, O.S.B. Al-Amoudi, A review on the angle of repose of granular materials, Powder Technol. 330 (2018) 397–417.
[27] P. Venkatesan, R. Manavalan, K. Valliappan, Preparation and evaluation of sustained release loxoprofen loaded microspheres, J. Basic Clin. Pharm. 2 (3) (2011) 159.
[28] H. Friedl, S. Dünnhaupt, F. Hintzen, C. Waldner, S. Parikh, J.P. Pearson, M.D. Wilcox, A. Bernkop-Schnürch, Development and evaluation of a novel mucus diffusion test system approved by self-nanoemulsifying drug delivery systems, J. Pharmaceut. Sci. 102 (12) (2013) 4406–4413.
[29] B. Mendes, C. Marques, I. Carvalho, P. Costa, S. Martins, D. Ferreira, B. Sarmento, Influence of glioma cells on a new co-culture in vitro blood–brain barrier model for characterization and validation of permeability, Int. J. Pharm. 490 (1-2) (2015) 94–101.
[30] J. Guillen, FELASA guidelines and recommendations, JAALAS 51 (3) (2012) 311–321.
[31] C.J. Morris, Carrageenan-induced paw edema in the rat and mouse, Inflammation protocols (2003) 115–121.

[32] A. Kumar, G.S. Chauhan, Extraction and characterization of pectin from apple pomace and its evaluation as lipase (steapsin) inhibitor, Carbohydrate Polymers 82 (2010) 454–459.
[33] P. Markov, S. Popov, I. Nikitina, R. Ovodova, Y.S. J.R. J.o.B.C. Ovodov, Anti-inflammatory activity of pectins and their galacturonan backbone 37 (7) (2011) 817–821.
[34] K. Cheewatanakornkool, S. Niratisai, S. Manchun, C.R. Dass, P. Sriamornsak, Thiolated pectin–doxorubicin conjugates: synthesis, characterization and anticancer activity studies, Carbohydrate polymers 174 (2017) 493–506.
[35] E.A. Almeida, S.P. Facchi, A.F. Martins, S. Nocchi, I.T. Schuquel, C.V. Nakamura, A.F. Rubira, E.C. Muniz, Synthesis and characterization of pectin derivative with antitumor property against Caco-2 colon cancer cells, Carbohydrate Polymers 115 (2015) 139–145.
[36] S. Khalid, G. Abbas, M. Hanif, S. Shah, S.N.H. Shah, A. Jalil, M. Yaqoob, N. Ameer, A. Anum, Thiolated sodium alginate conjugates for mucoadhesive and controlled release behavior of metformin microspheres, Int. J. Biol. Macromol. 164 (2020) 2691–2700.
[37] K. Kafedjiiski, R.K. Jetti, F. Föger, H. Hoyer, M. Werle, M. Hoffer, A. Bernkop-Schnürch, Synthesis and in vitro evaluation of thiolated hyaluronic acid for mucoadhesive drug delivery, International journal of pharmaceutics 343 (2007) 48–58.
[38] J. Rohrer, A. Partenhauser, O. Zupančič, G. Leonavičiūtė, S. Podričnik, A. Bernkop-Schnürch, Thiolated gelatin films: renaissance of gelatin as sustained intraoral dosage form, Eur. Polym. J. 87 (2017) 48–59.
[39] S. Majzoob, F. Atyabi, F. Dorkoosh, K. Kafedjiiski, B. Loretz, A. Bernkop-Schnürch, Pectin-cysteine conjugate: synthesis and in-vitro evaluation of its potential for drug delivery, J. Pharm. Pharmacol. 58 (2006) 1601–1610.
[40] B. Satheeshababu, R. Shruthinag, Synthesis and charactersiation of chitosan conjugate; design and evaluation of membrane moderated type transdermal drug delivery system, Indian J. Pharmaceut. Sci. 77 (4) (2015) 405.
[41] I. Khalid, M. Ahmad, M.U. Minhas, K. Barkat, Synthesis and evaluation of chondroitin sulfate based hydrogels of loxoprofen with adjustable properties as controlled release carriers, Carbohydrate polymers 181 (2018) 1169–1179.
[42] B. Satheeshababu, K. Shivakumar, Synthesis of conjugated chitosan and its effect on drug permeation from transdermal patches, Indian J. Pharmaceut. Sci. 75 (2) (2013) 162.
[43] V. Puri, A. Sharma, P. Kumar, I.J.P. Singh, Thiolation of biopolymers for developing drug delivery systems with enhanced mechanical and mucoadhesive properties, A review 12 (8) (2020) 1803.
[44] B. Robinson, L. Dalton, Monte Carlo statistical mechanical simulations of the competition of intermolecular electrostatic and poling-field interactions in defining macroscopic electro-optic activity for organic chromophore/polymer materials, J. Phys. Chem. 104 (20) (2000) 4785–4795.
[45] T. Ugurlu, M. Turkoglu, U.S. Gurer, B.G. J.E. J.o.P. Akarsu, Biopharmaceutics, Colonic delivery of compression coated nisin tablets using pectin/HPMC polymer mixture 67 (1) (2007) 202–210.
[46] K. Cheewatanakornkool, S. Niratisai, S. Manchun, C.R. Dass, P. Sriamornsak, Characterization and in vitro release studies of oral microbeads containing thiolated pectin–doxorubicin conjugates for colorectal cancer treatment, Asian J. Pharm. Sci. 12 (6) (2017) 509–520.