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Article

Cholic Acid-Grafted Thiolated Chitosan-Enveloped Nanoliposomes for Enhanced Oral Bioavailability of Azathioprine: In Vitro and In Vivo Evaluation

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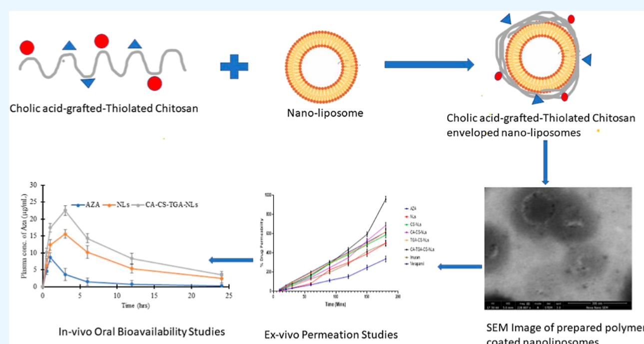
ABSTRACT: The purpose of the present study was to develop a cholic acid-grafted thiolated chitosan (CA-CS-TGA) polymeric biomaterial for attaining improved permeation via attaching thiol groups and cholic acid moieties. For this purpose, a CA-CS-TGA graft was prepared, and modification was confirmed via FTIR analysis. The prepared CA-CS-TGA graft was used to coat the azathioprine-loaded nanoliposomes (ENLs), with subsequent characterization in terms of zeta size, zeta potential, and SEM analysis. Pharmaceutical evaluation was carried out in terms of drug release studies, and ex vivo permeation and in vivo oral bioavailability were studied. The particle size and zeta potential of CA-CS-TGA coated nanoliposomal formulation CA-CS-TGA-NLs were found to be 245 ± 15.6 and +22.4 ± 0.58, respectively, compared to that of nonenveloped nanoliposomal formulation 165.7 ± 12.3 and −21.8 ± 0.14, respectively, indicating successful coating. CA-CS-TGA-NLs indicated 64% of drug release in 24 h at pH 7.4. Ex vivo permeation enhancement and relative oral bioavailability studies indicated a 2.84-fold enhanced permeation and 6-fold enhanced oral bioavailability of CA-CS-TGA-NLs compared to Azathioprine suspension. Based on the results, it can be concluded that grafting the CA-CS-TGA polymer onto nanoliposomes seems to be a promising strategy to enhance the oral bioavailability of Azathioprine.



■ INTRODUCTION

Azathioprine (AZA) is an immunosuppressive antimetabolite prodrug, a derivative of 6-mercaptopurine.[1] Immunomodulatory therapy with 6-mercaptopurine or azathioprine has become a choice of treatment for rheumatoid arthritis, Crohn's disease (CD), inflammatory bowel disease, and in the prevention of renal transplant rejection. However, its BCS-IV classification, characterized by its low solubility and decreased permeability, makes it difficult to formulate into a dosage form with optimum oral bioavailability, as the marketed tablets claim only 41−47% of the dose reaching the systemic circulation.[2] The inter- and intrasubject pharmacokinetic variations and side effects associated with high-doses jeopardize optimum therapeutic outcomes and patient compliance. In addressing the decreased oral bioavailability issue of AZA, various physicochemical factors like solubility, permeability, stability, and physiological barriers like transmembrane efflux via P-gp efflux pumps need to be targeted with an advanced drug delivery system.

Nanomedicine combined with smartly engineered polymeric excipients provides opportunities to explore and interact with the physicochemical and physiological barriers that hinder the optimum oral absorption of the drug. Nanoliposomes are best known for carrying hydrophobic drugs and crossing membranes effectively due to their close resemblance to the cell membrane structure and ability to form mixed micelles. However, the oral delivery of nanoliposomes is widely questioned due to the degradation of their constituting lipids by the gastric acids and the lipases present in the intestine.[3] Therefore, to utilize the full potential of nanoliposomes, degradation must be controlled before reaching the target site. In this regard, coating the nanoliposomes with polymeric excipients has been proven to be effective. The use of polymeric excipients, apart from protecting the lipids, can provide other opportunities for pharmaceutical interests like mucoadhesion and permeation enhancement.[4]

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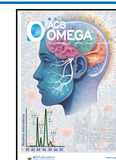

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The thiol-modified polymers, also known as thiomers, has been introduced to drug delivery systems in the recent past due to disulfide cross-linking, mucoadhesion, P-gp inhibition, and permeation-enhancing abilities. The thiol groups (−SH) provide a suitable method for achieving cross-linking by disulfide exchange mechanism via mild oxidizing agents without the use of a specific cross-linker.[5] These −SH groups are also involved in developing covalent bonds with the cysteine residue of the mucus, providing mucoadhesion, permeation enhancement due to the opening of the tight junction, and P-gp efflux pump inhibition.[6] Thus, a single thiolated polymer can serve multiple functions with high pharmaceutical aspects. Cholic acid-modified polymers are another type of polymeric excipient that can enhance oral permeation via apical sodium dependent bile acid transporters (ASBT). Following active transport via ASBT bile acids bind to intracellular bile acid binding proteins (IBABP) and then exit the enterocytes by the organic solute transporters OST-α and OST-β.[7] By keeping in view the above discussion, it would be a suitable strategy to develop AZA-loaded nanoliposomes coated with cholic acid-grafted thiolated polymer, where the thiolated polymer will develop a shell around the liposomes via disulfide cross-linked matrix, thus protecting the nanoliposomes from premature digestion in the gastric and intestinal fluids along with permeation-enhancing capabilities. Cholic acid-grafted moieties might target the ASBT mechanism, opening another gateway for enhanced permeation.

Therefore, in this study, thiolation of the chitosan was carried out with subsequent modification with cholic acid. The developed polymeric graft was then used as a coating material for the AZA-loaded nanoliposomes. The developed coated nanoliposomal formulations were then evaluated in terms of various pharmaceutical aspects, including the in vitro and in vivo evaluation of enhanced permeation and oral bioavailability, respectively.

■ MATERIALS AND METHODS

Synthesis of Thiolated Chitosan (CS-TGA). 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide) (EDAC)-mediated coupling method was used for the thiolation of chitosan[8] by coupling the chitosan (CS) with thioglycolic acid (TGA) via amide bond formation. Chitosan (1%, w/v) solution was prepared in acetic acid (1% v/v) with stirring, followed by the addition of 1 mL of TGA, 50 mM EDAC, and hydroxylamine (50 mM). The mixture's pH was adjusted to pH 4 using 0.1 M HCl solution, and the mixture was stirred for 4 h. To obtain purified CS-TGA and to remove unreacted materials from this solution, this mixture was dialyzed for 3 days in a dialyzing membrane (MW cutoff value 12−14 kDa) in the dark. Dialysis was carried out with a 5 mM HCl solution at first; then, this solution was dialyzed two times again with the same medium having 1% NaCl w/v and, at last, two times with 1 mM HCl so that the solution pH was adjusted to 4. After that, the purified thiolated chitosan (CS-TGA) solution was frozen at −80 °C, lyophilized, and stored at 4 °C until further use.

Synthesis of Cholic Acid-Grafted Thiolated Chitosan (CA-CS-TGA). Cholic acid was grafted onto the CS-TGA by the EDAC coupling method. First of all, 1 g of prepared TGA was dissolved in 46 mL of acetic acid solution (0.1 M) followed by the addition of 46 mL of ethyl alcohol. In a separate beaker, 2.17 mmol of cholic acid was dissolved in 10 mL of absolute ethyl alcohol, followed by the addition of 3.23 mmol of EDAC, and the mixture was stirred for 30 min at room temperature. This mixture was then added to the CS-TGA solution with continuous stirring at room temperature for 24 h. Cholic acid grafted thiolated chitosan (CA-TGA-CS) was then purified by dialyzing it for 3 days against 5 mM HCl. Purified CA-CS-TGA was frozen at −80 °C, lyophilized, and stored at 4 °C until further use.[9]

Determination of Stabilized Thiol Groups. The number of thiol groups attached to the chitosan backbone was quantified by using Ellman's reagent spectrophotometrically.[10] 250 μL of a 1% solution of CS, CS-TGA, and CA-CS-TGA were taken and added to 250 μL of phosphate buffer (pH 8.0) separately. 500 μL of Ellmen's reagent was added to these suspensions separately (Ellmen's reagent was prepared freshly by dissolving 40 mg in 10 mL of phosphate buffer of pH 8.0) and incubated for 3 h at room temperature. These samples were centrifuged at 15,000 rpm for 5 min, and the supernatant was removed carefully and transferred to the microwell plate. The absorbance was measured at 430 nm with a microtiter-plate reader. The TGA standard curve was used for the calculation of thiol groups on polymer grafts.[11]

Determination of Disulfide Bonds. To quantify the total amount of thiol groups present on thiolated chitosan and CS-TGA, the disulfide content was determined by the reduction of disulfide bonds by sodium borohydrate ($NaBH_4$). Disulfide bonds were formed due to the oxidation of the thiol group (SH-group). Disulfide content was measured by the reduction of these thiol groups by $NaBH_4$ and then the addition of Ellmen's reagent.[12] 350 μL of thiolated chitosan and CS-TGA were taken separately, and 650 μL of phosphate buffer (pH 6.8) was added to these solutions and incubated for 30 min. To these suspensions, freshly prepared sodium borohydride solution (1% $NaBH_4$) was added separately, and the resultant suspensions were incubated for 1 h at 37 °C. After this, to further decompose the remaining $NaBH_4$, 200 μL of HCl was added to them and stirred for 10 min. These solutions were neutralized by the addition of phosphate buffer (pH 8.0), followed by the addition of 100 μL Ellmen's reagent, and incubated for 1 h at room temperature. 250 μL of these solutions were removed carefully and transferred to a microplate, and their absorbance was measured at 430 nm using the microtiter-plate reader. The amount of disulfide bonds was determined by subtracting the calculated total thiol groups after the addition of $NaBH_4$ from the free thiol groups immobilized or stabilized on the modified polymers calculated in the previous section.[13]

Synthesis of Nanoliposomes (NLs). A dry film rehydration technique was used to prepare nanoliposomes.[14] 50 mg of lipid mixture was prepared to have cholesterol (25 mg) and phosphatidylcholine (25 mg) dissolved in an organic phase with chloroform and methanol (2:1, v/v), followed by the addition of 10 mg of azathioprine (AZA). Then, the dried thin lipid film was obtained after removing the organic phase in the rotary evaporator by maintaining the temperature of the water bath at 60 °C. The resultant produced film was dried under a vacuum and then rehydrated with phosphate buffer (pH 7.4) for 15 min to produce multilamellar vesicles with repeated vertexing. This suspension was sonicated on a bath sonicator for 20 min at 60 °C to further reduce the size of nanoliposomes. This nanoliposomal suspension was passed through an extruder to reduce the size of multilamellar vesicles. Nanoliposomes were freeze-dried and stored at −20 °C until needed in further studies.

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Synthesis of Enveloped Nanoliposomes. The weighed amount of azathioprine-loaded nanoliposomes (AZA-NLs) was suspended in 1% w/v solutions of CS, CS-TGA, CA-CS, and CA-CS-TGA separately and kept stirring for 5 h to stabilize the coating of CS and graft polymers onto AZA-NLs via the electrostatic interaction to produce CS-NLs, CA-CS-NLs, TGA-CS-NLs, and CA-CS-TGA-NLs. These coated, enveloped nanoliposomes were freeze-dried and stored at −20 °C until further use.[15]

FTIR, Zeta Potential, Particle Size, and Surface Morphology Evaluation. The grafting of the polymeric excipients and the compatibility of the drug with the prepared grafts were analyzed through Fourier transformed infrared (FTIR) spectroscopy using an ATR-FTIR Spectrophotometer (Bruker alph-P, USA). FTIR analysis was carried out on CS, CS-TGA, and CA-CS CA-TGA-CS to confirm the grafting.

Particle size and zeta potential of prepared blank NLs and enveloped nanoliposomes, CS-NLs, CA-CS-NLs, TGA-CS-NLs, and CA-TGA-CS-NLs, were evaluated through a zetasizer (Malvern, Nano ZSP, UK). A scanning electron microscope (FEI Nova NanoSEM 450, USA) equipped with a transmission electron detector operated at 17.5 kV was used to determine surface morphology.

Entrapment Efficiency. The entrapment efficiency of the prepared nanoliposomal formulations was checked by using the centrifugation method, in which the freshly prepared liposomal formulations were subjected to centrifugation at 15,000 rpm for 30 min, and the temperature was maintained at 4 °C. The supernatant was collected, diluted with DMSO, and evaluated for the amount of untrapped AZA via analysis by UV-spectroscopy at 276 λ_{max}.[16] The entrapment efficiency was calculated by following the formula

$$\text{entrapment efficiency (\%)} = \frac{\text{(amount of drug informulation)}}{\text{total amount of drug}} \times 100$$

$$\text{amount of drug informulation} = \text{total drug loaded in liposomes} - \text{unentrapped drug}$$

% Water Absorbing Capacity. Water-absorbing capacity was evaluated for azathioprine-loaded nanoliposomes, CS-NLs, CA-CS-NLs, CS-TGA-NLs, and CA-CS-TGA-NLs. Thin tablets of 5 mm from each formulation were developed in the lab scale tablet punching machine by pressing the lyophilized powder of each formulation. These tablets were fixed on the tip of the needle and immersed in acidic (at pH 2) and basic medium (at pH 7.4) to check their absorbing capacity in both mediums separately at 37 °C. After a predetermined time interval, the tablets were removed from the medium, and excess water was removed from the tablet surface with the help of tissue paper and weighed. The water uptake capacity by these formulations was calculated by the gravimetric formula given below[10]

$$\text{water absorbing capacity (\%)} = \frac{\text{Wf} - \text{Wo}}{\text{Wo}} \times 100$$

where Wo = initial weight and Wf = weight of the hydrated tablet.

Mucoadhesion Study. A mucoadhesion study was performed to check the residence time of prepared nanoliposomal formulations with mucin of the intestinal membrane. For this purpose, goat intestine was obtained from the slaughterhouse, washed, and placed in a normal saline solution. A piece of intestine was then mounted on a basket-tube rack of disintegration apparatus covered entirely with aluminum foil, double tape, and the basal side of the mucosa contacting immersion fluid (pH 7.4) present in the chamber of the disintegration apparatus. Compressed tablets of nanoliposomal formulation AZA-NLs and coated nanoliposomal formulations CS, CS-TGA, CA-CS, and CA-TGA-CS were compressed into tablets of 5 mm, and three tablets of each formulation were placed onto the intestinal mucosa by gently pressing against the intestine. After starting the disintegration apparatus, the time was noted at which the tablet detached from the mucous membrane. This was done for all other formulations by noting their time of detachment. Detachment from the mucous membrane predicted its time of residence with the mucous membrane.

In Vitro Drug Release. To study in vitro drug release from nanoliposomes (NLs) and enveloped nanoliposomes (ENLs), CS, CS-TGA, CA-CS, and CA-TGA-CS, the dialysis membrane diffusion method was used.[17] The dialysis membrane (MW cutoff value 12−14 kDa) was soaked overnight, and the weighed quantity of formulations having drug equivalent to 10 mg was placed in that dialysis membrane and sealed from ends. These dialysis membranes were immersed in phosphate buffer (pH 2−7.4) separately in dissolution apparatus II. These dialysis bags were hung with the paddle in medium (500 mL in each vessel) having either acidic medium or basic medium (separately done for both mediums). The system was maintained at 37 °C ± 0.5 and 50 rpm. Samples were collected at predefined time intervals and analyzed spectrophotometrically (UV spectrophotometer) at 276 λ_{max}. For the acidic medium, the study was performed for 3 h, and for the basic medium, the study was performed for 24 h. The drug release data were later analyzed with DDSolver, a Microsoft Excel Add-in, to evaluate the release kinetics of these formulations.

Ex Vivo Permeation Study. A Franz diffusion cell was used for the determination of ex vivo permeation of the prepared nanoliposomal formulations.[18] Franz diffusion cell apparatus consisting of two-limbed reservoirs with an effective diffusion area of 1.15 cm^2. The donor compartment was about 15 mm in diameter at the orifice, having a capacity of about 2 mL, and the receptor compartment had a capacity of about 9 mL. The intestine of the goat was taken freshly and washed thoroughly with Krebs ringer lactate solution (pH 7.4). The intestine was cut into pieces of 4−5 cm. The piece of intestine was placed on the donor cell in such a way that the mucus layer was facing upward toward the receptor cell, fixed well, and the receptor cell was then placed and fixed properly over the mucous layer of the intestine. The receptor cell was filled with 9 mL of phosphate buffer (pH 7.4) and stirred continuously at 50 rpm. The temperature of the whole system was maintained at 37 °C. Nonenveloped nanoliposomes equivalent to 5 mg of AZA were placed in the donor part of the Franz-diffusion cell separately. AZA pure drug solution and Imuran tablet suspension equivalent to 5 mg of AZA were also added in separate Franz-diffusion cells. The samples of 0.5 mL were withdrawn from receptor cells at a predetermined time interval and also replaced with fresh medium to maintain sink condition. The study was performed for 3 h. The amount of drug was quantified through a validated HPLC method reported previously. Quantification was carried out using an

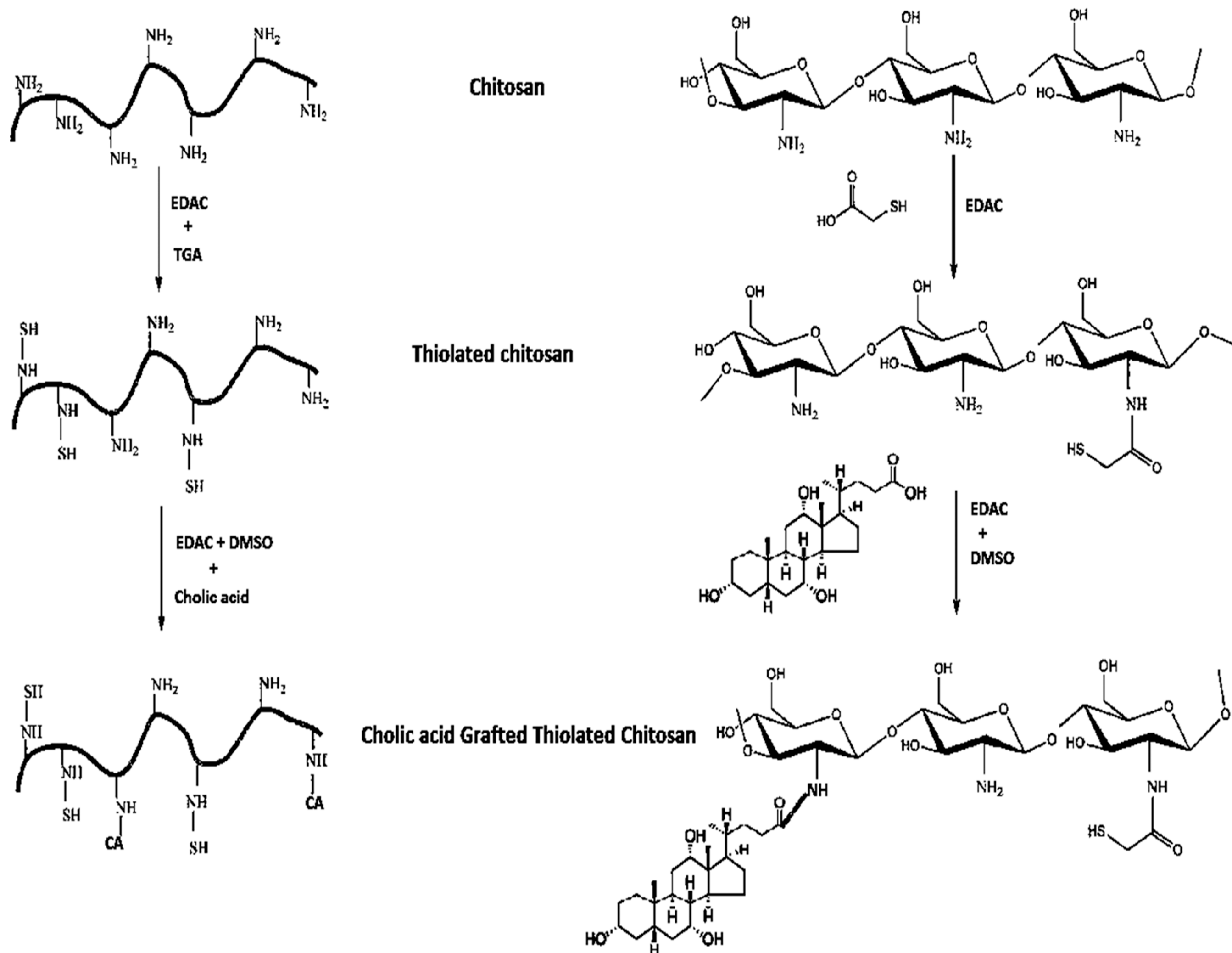

Figure 1. Synthetic pathway of cholic acid-grafted thiolated chitosan with thiolation via the EDAC coupling method.

RP-C_{18} stainless steel column (250 × 4 mm in diameter with 5 μm particle size and packing material Euro sphere-100). The isocratic mode was used with mobile phase containing acetonitrile, water, and methanol in a ratio of 25:70:05 and pH adjusted to 4 ± 0.1 using glacial acetic acid at a flow rate of 1.0 mL/min and an injection volume of 20 μL. The detection wavelength was set at 280 nm and the linearity was found to be in the range of 30−90 μg/mL with an R^2 value of 0.999.

The apparent permeability was then calculated by using the following equation

$$P_{app} = (dQ/dt)/(C^0 \times A)$$

where (dQ/dt) is the transport rate or flux (J) (μg/min) across the biological membrane, C^0 (μg/mL) is the initial concentration of the drug in the donor chamber, and A is the surface area (cm^2) of the permeation membrane.

In Vivo Bioavailability Studies. In vivo bioavailability studies were performed to check the improved pharmacokinetic parameters of azathioprine. The enveloped liposomal formulation CA-TGA-CS-NLs was selected to perform the oral bioavailability studies because of its maximum ex vivo permeation, along with nonenveloped NLs and AZA drug suspension for comparison purposes. For this purpose, animal studies were conducted by following the protocol guidelines approved by the research ethics committee of the Riphah Institute of Pharmaceutical Sciences, Riphah International University, Lahore Campus, with approval reference no. REC/2021/447. Healthy Wistar rats of 6 to 8 weeks were taken, weighing 200 ± 20 g. They were kept in the animal house and given free access to food and water. The rats were kept fasting for 12 h a day before experimenting on them. The rats were divided into 4 main groups (n = 24 in each group). Group 1 was treated with a pure azathioprine (AZA) suspension in pure water. Group 2 was given NLs Group 3 was treated with enveloped nanoliposomes CA-TGA-CS-NLs, and Group 4 served as the control. All the groups were given a dose equivalent to 3 mg/kg of body weight by using an oral gavage needle. At each sampling time point (0.5, 1, 3, 6, 12, and 24 h) 4 rats were randomly selected from each treatment group and euthanized using a CO_2 chamber. About 7−8 mL of blood samples were withdrawn from the direct cardiac puncture of rats. These blood samples were transferred to 2 mL of Eppendorf with 100 μL of an anticoagulant (11% sodium citrate). These samples were immediately centrifuged at 4000 rpm for 15 min, and plasma was separated and stored at −20 °C until further use for analysis. From the collected plasma samples, drugs were extracted and analyzed through a validated HPLC method.[19]

Statistical Analysis. Statistical analysis was carried out by using ANOVA followed by Tukey's post hoc test for multiple comparisons, while a *t*-test was carried out for the calculation of significant differences between two observations. All the tests were applied by using GraphPad Prism.

■ RESULTS AND DISCUSSION

The synthesis of a cholic acid graft on thiolated chitosan by modification of the chitosan backbone achieved by the introduction of thiol groups was expected to enhance the oral bioavailability and permeability of a class IV drug, i.e., Azathioprine (AZA). The AZA was enclosed in nanoliposomes that were supposed to deliver hydrophobic drugs by preventing their environmental degradation. The graft was supposed to improve permeation through intestinal epithelium and P-gp efflux pump inhibition, thus have to enhancing the therapeutic effectiveness of azathioprine. Different experiments were designed and conducted to support the aims and objectives of this study.

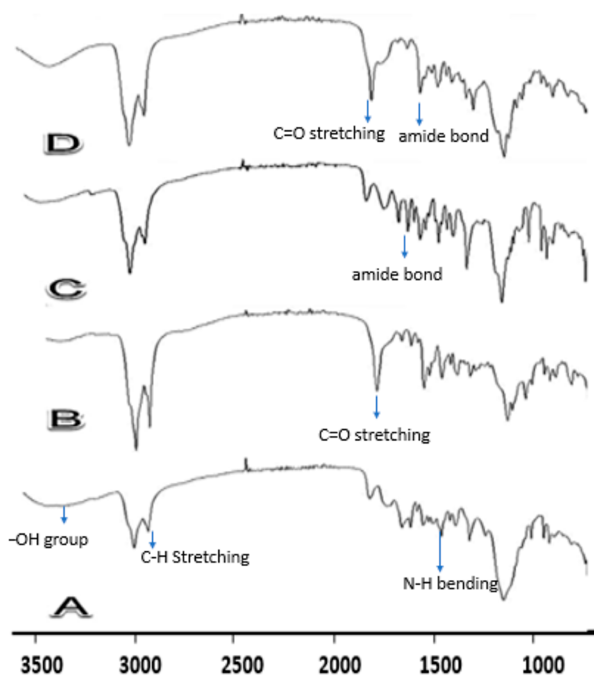

Figure 2. FTIR spectra of (A) chitosan (CS), (B) cholic acid-chitosan graft (CA-CS), (C) thiolated chitosan graft (CS-TGA), and (D) cholic acid-thiolated chitosan graft (CA-CS-TGA).

Table 1. Characterization of Zeta Potential, Particle Size, and PDI of NLs, CS-NLs, CA-CS-NLs, TGA-CS-NLs, and CA-TGA-CS-NLs

formulations	zeta potential (mv)	particle size (nm)	PDI
NLs	−21.8 ± 0.14	165.7 ± 12.3	0.21 ± 0.01
CS-NLs	+32.6 ± 0.22	180.2 ± 14.1	0.29 ± 0.01
CA-CS-NLs	+23.8 ± 0.36	187.3 ± 11.7	0.25 ± 0.02
TGA-CS-NLs	+39.6 ± 0.53	200.5 ± 9.8	0.24 ± 0.01
CA-TGA-CS-NLs	+22.4 ± 0.58	245 ± 15.6	0.27 ± 0.05

Synthesis and Characterization of Thiolated Chitosan. Thiolated chitosan was synthesized successfully by modification of the chitosan backbone. Thiolation was carried out via a carbodiimide condensation reaction between chitosan and thioglycolic acid. The EDAC coupling mechanism led to the formation of an amide bond between the −COOH group of thioglycolic acid (TGA) and the $-NH_2$ group of chitosan, as shown in Figure 1. The lyophilized thiolated chitosan appeared as a white powder with fibrous flakes. The determination of the quantification of immobilized thiol groups and disulfide bonds was done through Ellmen's reagent. The immobilized thiol groups in thiolated chitosan were 624 μM/g. The amount of disulfide bonds was 128 ± 73 μM/g.

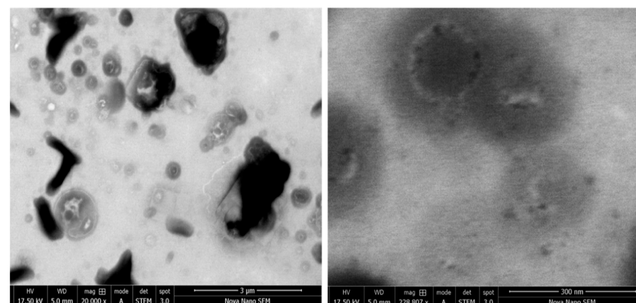

Figure 3. SEM images of the enveloped nanoliposomal formulation based on thiolated cholic acid-grafted chitosan CA-TGA-CS-NLs at different magnifications.

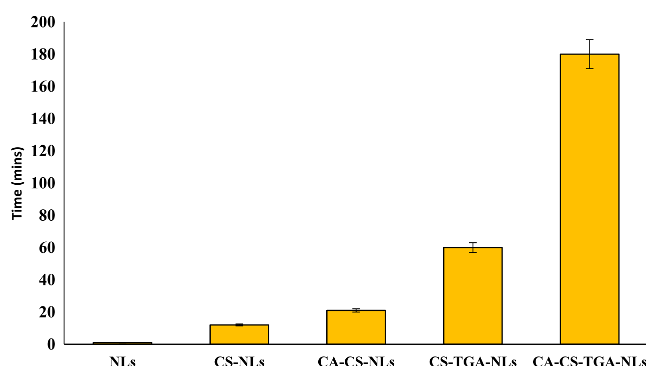

Figure 4. Mucoadhesion study of NLs, CS-NLs, CA-CS-NLs, CS-TGA-NLs, and CA-CS-TGA-NLs. The results are the mean ± SD of 3 experiments.

Synthesis and Characterization of Cholic Acid-Grafted Thiolated Chitosan. The synthesis of a graft of cholic acid on thiolated chitosan was carried out in the same manner as the EDAC coupling mechanism through the carbodiimide reaction. Covalent linkage was formed between the carboxylic group (−COOH) of cholic acid and the amino group ($-NH_2$) of thiolated chitosan. The determination of the quantification of immobilized thiol groups and disulfide bonds was done through Elman's reagent. The immobilized thiol groups in cholic acid grafted thiolated chitosan were 624 μM/g. The amount of disulfide bonds was 128 ± 73 μM/g. The pH was maintained at pH 4 during the synthesis of the polymeric grafts to prevent disulfide bond formation, and dialysis was also carried out in the dark to prevent oxidation of thiol groups.

FTIR Analysis. The FTIR spectrum was performed to evaluate confirmation of the modified grafted polymer coated on nanoliposomes loaded with AZA, as shown in Figure 2. The characteristic peaks of chitosan graft at 3362.90, 2924.27, 1331.85, and 1151.31 cm^{-1} represented −NH vibrations, C−H stretching, S=O stretching, and C−O strong stretching. Thiolated chitosan grafts showed successful characteristic

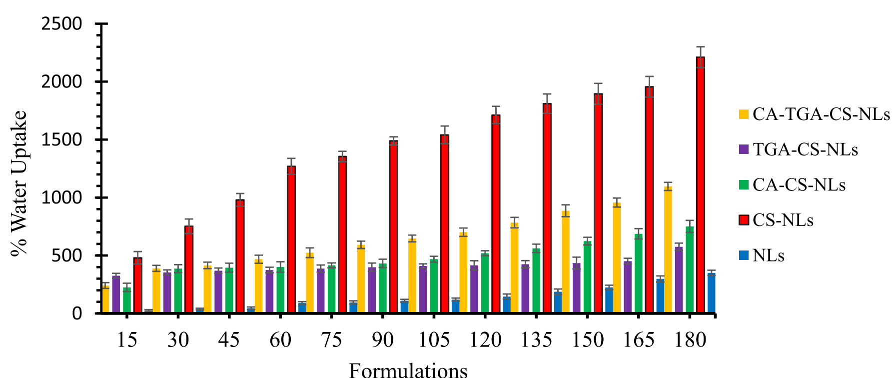

Figure 5. % Water uptake of NLs, CS-NLs, CA-CS-NLs, CS-TGA-NLs, and CA-CS-TGA-NLs. The analysis was carried out in phosphate buffer at pH 7.4 for 3 h. The results are the mean ± SD of 3 experiments.

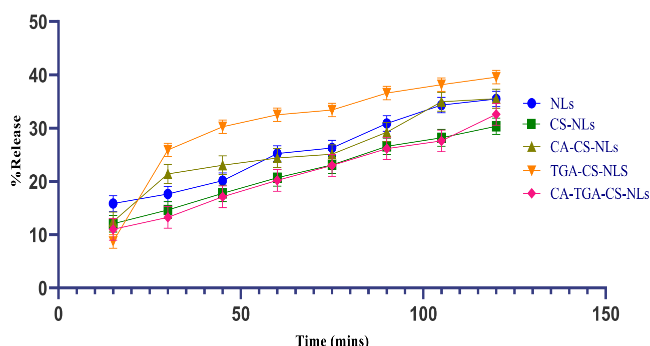

Figure 6. Drug release mechanism of AZA-loaded NLs, CS-NLs, CA-CS-NLs, CS-TGA-NLs, and CA-CS-TGA-NLs. Dissolution studies were carried out at pH 2. The results are the mean ± SD of 3 experiments.

peaks at 3375.66 cm^{-1} (OH stretching), 2925.96 cm^{-1} (C–H stretching), 1650.46 cm^{-1} (C=C stretching), and 1528.87 cm^{-1} (N–O stretching). Cholic acid-chitosan grafts showed successful characteristic peaks at 3305.55 cm^{-1} (OH stretching), 2916.81 cm^{-1} (C–H stretching), and 1703.04 cm^{-1} (strong carbonyl stretching). The FTIR spectrum of Cholic acid-thiolated chitosan graft showed a characteristic peak at 3327.10 46 cm^{-1}, representing NH, and OH stretching showing amide linkage ,then at 1708.346 cm^{-1} representing strong carbonyl stretching (C=O), 1530.30 showing (N–H) stretching. Peaks in both the Cholic acid-chitosan graft and the Cholic acid-thiolated chitosan graft at 1703.04 and 1708.30 cm^{-1}, respectively, showed a characteristic band for strong carbonyl conjugated acid (incorporation of the carboxylic group of cholic acid). Peaks in thiolated chitosan graft, cholic acid-chitosan graft, and cholic acid-thiolated chitosan graft at 1373.80, 1374.83, 1375.69 cm^{-1}, respectively, represent a modification of the $-NH_2$ group with a thiol group. Characteristics of amide bond formation, its conjugation with the thiol group, and then with carboxylic acid (−COOH) of cholic acid confirms the successful modification in the grafted polymer.

Synthesis of Nanoliposomes and Enveloped Nanoliposomes. A thin-film rehydration technique was used for the formation of both empty and loaded nanoliposomes with azathioprine. Cholic acid-grafted thiolated chitosan (CA-TGA-CS) was coated on nanoliposomes through the ionic interaction between the negatively charged lipid bilayer of liposomes and the positively charged grafted polymer (CA-TGA-CS), leading to the formation of ENLs. For the confirmation of successful ENLs, zeta potential and particle size were evaluated, and the results are presented in Table 1. The change of zeta potential from −21.8 ± 0.14 to +32.6 ±

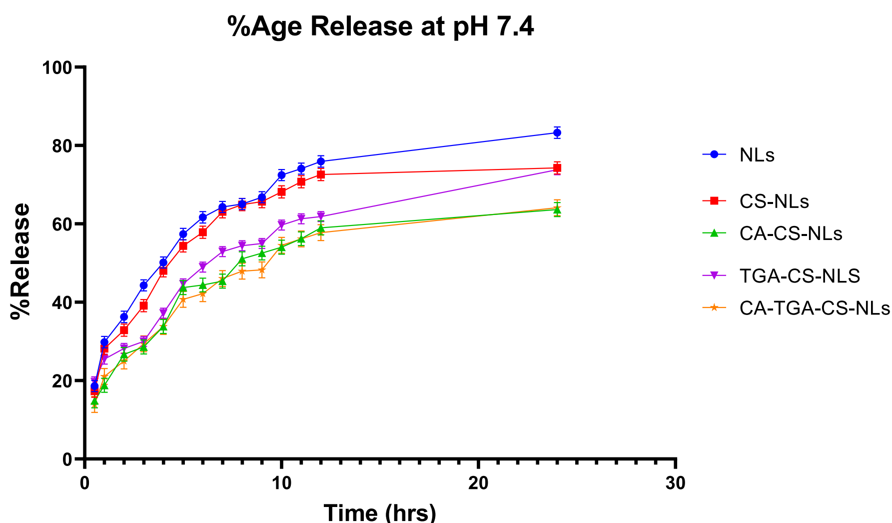

Figure 7. Drug release mechanism of AZA-loaded NLs, CS-NLs, CA-CS-NLs, CS-TGA-NLs, and CA-CS-TGA-NLs. Dissolution studies were carried out at pH 7.4. The results are the mean ± SD of 3 experiments.

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Table 2. Drug Release Data Modeling Based on the In Vitro Drug Release of AZA-Loaded NLs, CS-NLs, TGA-CS-NLs, and CA-TGA-CS-NLs to Determine the Possible Drug Release Mechanism at pH 7.4[a]

kinetic models	NLs		CS-NLs		CA-CS-NLs		TGA-CS-NLs		CA-TGA-CS-NLs	
	slope	R^2	slope	R^2	slope	R^2	slope	R^2	slope	R^2
zero-order	K_O = 5.808	0.929	K_O = 5.468	1.023	K_O = 4.381	0.483	K_O = 4.847	0.5281	K_O = 4.311	0.421
first-order	K_1 = 0.149	0.7845	K_1 = 0.135	0.7241	K_1 = 0.084	0.6372	K_1 = 0.098	0.6830	K_1 = 0.080	0.6404
Higuchi	KH = 21.95	0.7954	KH = 20.78	0.7392	KH = 16.428	0.8623	KH = 18.10	0.8765	KH = 16.117	0.8880
Korsmeyer-Peppas	kKp = 31.72	0.9548	kKp = 30.54	0.9149	kKp = 21.96	0.9439	kKp = 24.37	0.9648	kKp = 21.26	0.9610
	n = 0.336		n = 0.329		n = 0.371		n = 0.368		n = 0.377	

[a]N < 0 45 indicating the Fickian type of drug release behavior.

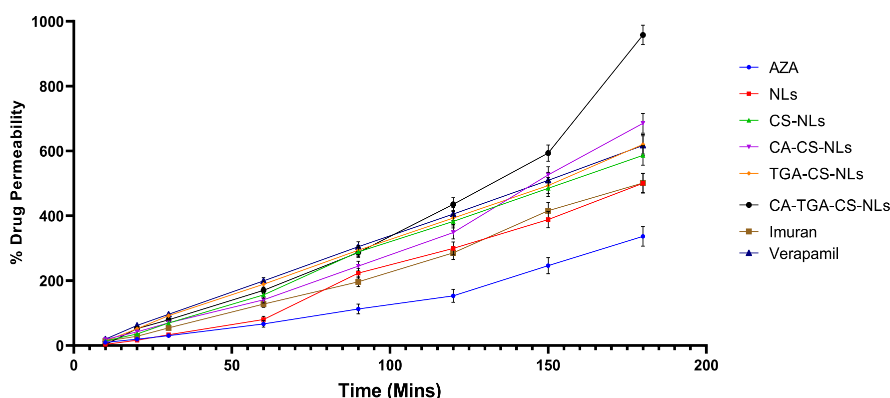

Figure 8. Permeation of Azathioprine in the presence of verapamil and synthesized NLs, CS-NLs, CA-CS-NLs, CS-TGA-NLs, and CA-CS-TGA-NLs. The results are the mean ± SD of 3 experiments.

Table 3. Results Showing Ex Vivo Permeation in Terms of Concentration Transported Across the Intestine and Apparent Permeability Along with the Improvement Ratio of Azathioprine in the Presence of Verapamil and Synthesized NLs, CS-NLs, CA-CS-NLs, CS-TGA-NLs, and CA-CS-TGA-NLs

formulations	apparent permeability coefficient (Papp ×10<sup>$^{-6}$ (cm))	enhancement ratio
AZA	6.7335	
NLs	10.005	1.485
CS-NLs	11.7285	1.7418
CA-CS-NLs	13.706	2.035
TGA-CS-NLs	12.413	1.8434
CA-TGA-CS-NLS	19.162	2.845
Imuran	10.029	1.489

0.22, +23.8 ± 0.36, +39.6 ± 0.53, and +22.4 ± 0.58 in the cases of CS-NLs, CA-CS-NLs, TGA-CS-NLs, and CA-TGA-CS-NLs, respectively, indicate successful coating via the electrostatic interaction. The maximum zeta potential was observed in the case of CS-NLs, and the decreased zeta potential of the behavior of formulations TGA-CS-NLs and CA-TGA-CS-NLs is due to the modification of the amino groups of chitosan with thiol groups and thiol and cholic acid, respectively, resulting in a decreased amount of ionizability. The SEM images, as shown in Figure 3, depict the round morphology of the CA-TGA-CS-NLs, where a distinct core and shell can be identified.

Mucoadhesion Study. Mucoadhesion is a key parameter for the mechanism of binding of nanoformulations, their penetration, cohesiveness, swelling behavior, and permeation across the intestinal membrane. Thiolated polymers are well-known for their mucoadhesive character due to their ability to develop disulfide bonds with the cysteine residue of the mucus.

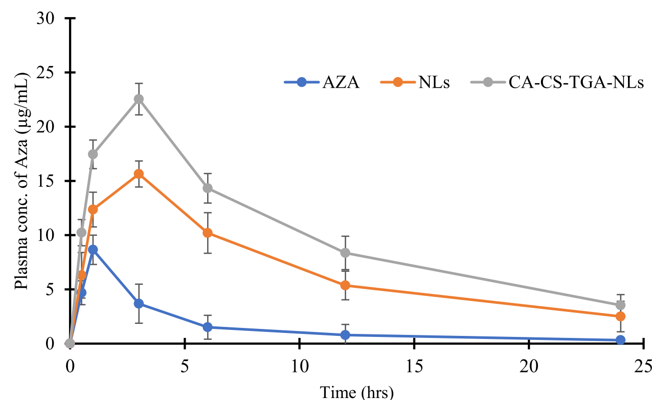

Figure 9. Comparative rat plasma concentration−time profile of AZA after administration of equivalent concentrations of AZA (3 mg/kg) loaded to the liposome and CA-CS-TGA nanoliposomes ad AZA suspension. Blood was collected from all groups at the indicated time points. AZA was extracted from plasma, and the concentration of AZA was determined by HPLC analysis.

One of the purposes of enveloping liposomes with thiolated polymers was to enhance their mucoadhesion properties, thus providing increased contact time of the mucus for better penetration to reach the intestinal cell surface. The mucoadhesion properties of NLs, CS-NLs, CA-CS-NLs, TGA-CS-NLs, and CA-TGA-CS-NLs are shown in Figure 4. Thiomers grafts coated NLs, i.e., TGA-CS-NLs and CA-TGA-CS-NLs, showed increased mucoadhesion time, i.e., 60 ± 7 and 180 ± 13 min, respectively, compared to CS-NLs and CA-CS-NLs with mucoadhesion times of 12 ± 3 and 21 ± 2 min, respectively. The increased mucoadhesion of TGA-CS-NLs and CA-TGA-CS-NLs may be attributed to the presence of thiol and cholic acid groups, providing increased interaction

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Table 4. Pharmacokinetic Parameters of Azathioprine-Loaded Nanoliposomes, Azathioprine-Loaded Colic Acid Grafted Thiolated Chitosan Nanoliposomes, and Azathioprine Obtained after Non-compartmental Analysis by PKSolver

parameters	formulations		
	AZA	NLs	CA-CS-TGA-NLs
dose (mg/kg)	3.0	3.0	3.0
$t_{1/2}$(h)	1.5	8.17	8.165
T_{max} (h)	1.00	3	3.0
C_{max} (μg/mL)	8.65	15.64	22.54
AUC_{0-24} (μg/mL h)	58.17	166.84	244.195
F^a (%)	15.14	43.22	63.54

[a]For relative oral bioavailability (F) calculation, $AUC_{i.v.}$ after administration of 3 mg of AZA per kg body weight was 384 ± 74 μg/mL h.

with the mucous via disulfide bonds and ionic interaction, respectively.

% Water Absorbing Capacity. Swelling studies were performed to evaluate the water-absorbing capacity of our prepared formulations, including NLs, CS-NLs, CA-CS-NLs, TGA-CS-NLs, and CA-TGA-CS-NLs, in a basic medium (at pH 7.4), as shown in Figure 5. Swelling plays an important role in the mucoadhesive drug delivery system, as the hydrated form of the polymers leads to mobility of the polymeric chains, thus providing room for interaction and interlocking with the mucus. However, the swelling behavior of the coating polymer must be optimized, as excessive swelling of the polymer may lead to the rapid degradation and loss of the coating along with the rapid release of the drug. CA-TGA-CS-NLs, CA-CS-NLs, and TGA-CS-NLs showed moderate and gradual swelling, which shows the controlled manner of drug release, while nonthiolated nanoliposomes, i.e., CS-NLs, showed higher swelling. The controlled swelling behavior of the thiolated polymer-enveloped nanoliposomes compared to that of chitosan-coated nanoliposomes is due to the disulfide cross-linked structure of the thiolated polymer at pH 7.4, which provides strength and controls swelling.

Drug Release Studies. Dissolution studies were performed for the evaluation of drug release. AZA released from grafted nanoliposomes was estimated by sink condition dialysis at pH 2 and pH 7.4, as shown in Figures 6 and 7, respectively. AZA loaded in TGA-CS-NLs showed higher release in an acidic medium, i.e., 30% of the drug was released during the first 45 min. It showed an abrupt release of drug, i.e., 26% after 15 min, as compared to other nonmodified chitosan-coated formulations. While only 17% of drugs released from CA-TGA-CS-grafted nanoliposomes This might be due to the reduction of disulfide bonds in the thiolated chitosan to produce thiol groups, thus breaking the cross-linking of the disulfide cross-linked thiolated chitosan, resulting in the ease of drug release. AZA release from NLs, CA-NLs was also greater than drug release from CA-TGA-CS-grafted nanoliposomes, as CA-TGA-CS showed sustained release by preventing AZA from an acidic environment. This might be due to the presence of acidic functional groups that remained mostly in unionized form at the acidic pH, providing limited swelling of the polymeric chains. In a basic medium (pH 7.4), CA-TGA-CS-grafted nanoliposomes maintained sustained release of AZA for 24 h, i.e., 64% of the drug had been released during 24 h, which showed its controlled release. CA-NLs also showed less release of AZA in the basic medium, i.e., 63% of the drug release. TGA-CS-NLs showed 73% release in the basic medium as compared to the acidic medium. For the estimation of drug release, various release kinetic models were applied. The model that was best suited for release data was evaluated using a high degree of correlation coefficient R square value. The results shown in Table 2 indicate that drug release from formulations followed the Korsmeyer-Peppas model based on an R^2 value indicating a Fickian-type drug release pattern at pH 7.4.

Ex Vivo Permeation Studies. Ex vivo permeation studies were carried out on Franz cells to estimate the permeation of AZA. Results of permeation studies for AZA are presented in Figure 8, and Papp values along with enhancement ratios are represented in Table 3. Verapamil is a well-known P-gp inhibitor and was used with AZA to provide proof of concept that the use of P-gp inhibition can enhance the oral bioavailability of a drug that is a substrate of P-gp efflux pumps. These results showed significantly enhanced permeation of AZA when used with verapamil as compared to AZA alone. Similarly, thiolated formulations, including CA-TGA-CS-NLs and TGA-CS-NLs, showed significant improvement in permeation because of their ability to inhibit P-gp efflux pumps. Furthermore, the thiolated polymers are also well-known for their permeation enhancement abilities to provide better penetration across the intestinal mucus layer due to their excellent mucoadhesion properties and the opening of tight junctions. The Papp enhancement ratios for CA-TGA-CS-NLs, TGA-CS-NLs, and CA-CS-NLs were found to be 2.84-fold, 1.84-fold, and 2.03-fold increased, respectively, representing a significant improvement in the permeation of AZA.

In Vivo Bioavailability Studies. Relative oral bioavailability and pharmacokinetic parameters of AZA, AZA-loaded NLs, and AZA-loaded CA-TGA-CS-NLs at different time intervals for 24 h after single dose oral administration were analyzed and are presented in Figure 9 and Table 4, respectively. From pharmacokinetic parameters, after oral administration, it was observed that AZA reached the Cmax of 8.65 (μg/mL) in 1 h, AZA-loaded NLs reached the Cmax of 15.64 (μg/mL) in 3 h, while AZA-loaded CA-TGA-CS-NLs reached the Cmax of 22.54 (μg/mL) in 3 h. The Cmax of AZA-loaded CA-CS-TGA-NLs showed a 2.6-fold increase as compared to that of pure AZA. The AUC_{0-24} of AZA-loaded CA-CS-TGA-NLs showed a 5.95-fold increase compared to that of pure AZA, reflecting almost a 6-fold increase in the relative oral bioavailability. The enhanced bioavailability in the case of AZA-loaded CA-CS-TGA-NLs may be attributed to the covalently attached thiol groups along with cholic acid moieties, leading to enhanced oral permeability.[20]

■ CONCLUSIONS

The present study successfully achieved the aim of enhancing the oral bioavailability of azathioprine by coating the thiolated cholic acid-grafted chitosan (CA-TGA-CS) onto azathioprine-loaded nanoliposomes. The nanoformulation of CA-CS-TGA-NLs was found to be 245 ± 15.6 with significantly enhanced mucoadhesion potential exhibiting 180 ± 13 min of mucoadhesion time. The in vitro release showed a prolonged cumulative release of about 64% for 24 h. Similarly, CA-CS-TGA-NLs exhibited 2.84-fold enhanced intestinal permeation compared to Azathioprine suspension. The AUC_{0-24} of AZA-loaded CA-CS-TGA-NLs showed a 5.95-fold increase compared to that of pure AZA in relative oral bioavailability.

The mucoadhesion was moderate, and permeation studies showed a 2.84-fold increase. Based on the results, it can be concluded that coating the thiolated cholic acid grafted chitosan (CA-TGA-CS) onto azathioprine-loaded nanoliposomes could be a potential approach to developing the formulation with enhanced oral bioavailability.

The prospects include detailed in vivo pharmacokinetic studies to describe the PK parameters necessary to develop the dosage of this drug delivery system, along with detailed toxicological profiling in animal models to ensure the safety of the developed nanoformulations.

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Notes

The authors declare no competing financial interest.

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