



Development of Eudragit-Based Nanofiber Formulations for Colon-Targeted Delivery of Hydrocortisone in Inflammatory Bowel Disease

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Abstract

Purpose Inflammatory bowel disease (IBD) is a chronic inflammatory disorder requiring effective localized drug delivery to minimize systemic side effects. This study aimed to develop pH-responsive, Eudragit-based nanofiber formulations for colon-targeted oral delivery of hydrocortisone (HC).

Methods HC-loaded nanofibers were fabricated using the electrospinning technique with Eudragit[®] S100 and Eudragit[®] L100-55 polymers. Physicochemical characterization was performed using Differential Scanning Calorimetry (DSC), Fourier Transform Infrared Spectroscopy (FT-IR), X-ray Diffraction (XRD), and Proton Nuclear Magnetic Resonance (¹H-NMR) analyses. Morphology, mechanical properties, contact angle, mucoadhesion, in vitro drug release, and ex vivo permeability were evaluated. Cytotoxicity and anti-inflammatory activity were assessed using RAW 264.7 macrophage cells.

Results XRD and DSC analyses indicated a reduction in the crystalline nature of HC within the nanofiber matrix, suggesting partial amorphization following electrospinning. The optimized formulation (E4) exhibited uniform morphology, suitable mechanical properties, and enhanced mucoadhesion. In vitro release studies demonstrated minimal drug release under gastric conditions (~11% at pH 1.2) and increased release at intestinal and colonic pH, confirming pH-responsive behavior. Ex vivo studies showed comparable permeability among formulations. Cell culture studies revealed that the formulations were non-cytotoxic (>80% cell viability) and significantly reduced TNF- α expression by approximately 39% at the protein level and showed a comparable reduction at the mRNA level.

Conclusion The developed Eudragit-based nanofiber system, particularly the E4 formulation, demonstrated effective colon-targeted delivery, favorable physicochemical properties, and significant anti-inflammatory activity. These findings indicate that HC-loaded nanofibers represent a promising platform for the localized treatment of IBD.

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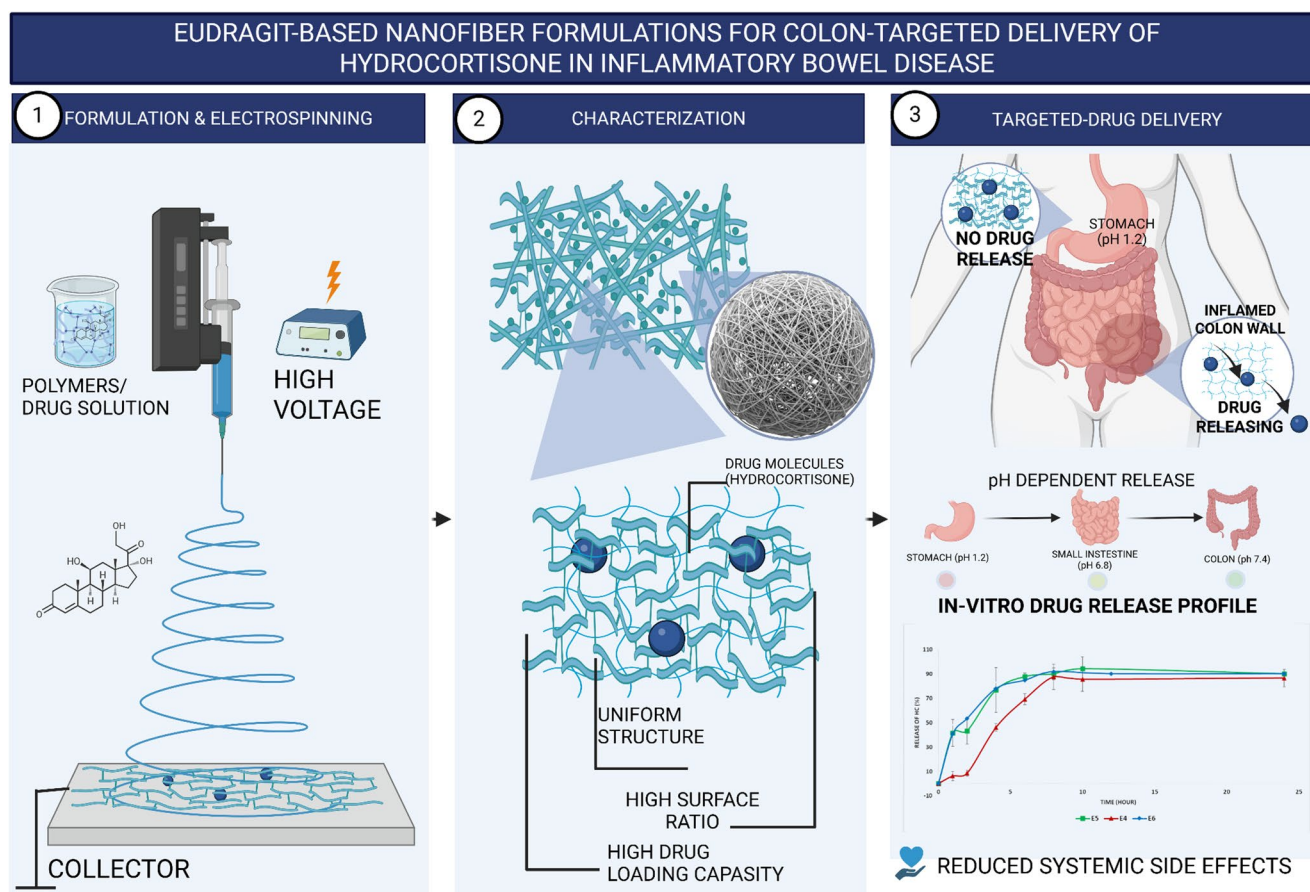
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Graphical Abstract



Keywords Hydrocortisone · Nanofiber · Inflammatory bowel disease · Electrospinning

Abbreviations

DSC	Differential Scanning Calorimetry
FT	IR–Fourier Transform Infrared Spectroscopy
SEM	Scanning Electron Microscopy
XRD	X-ray Diffraction
NMR	Nuclear Magnetic Resonance
TNF	α -Tumor Necrosis Factor- α
PCR	Polymerase Chain Reaction
IBD	Inflammatory Bowel Disease
HC	Hydrocortisone
DMF	Dimethylformamide
EtOH	Ethanol

Introduction

Inflammatory bowel disease (IBD) is a chronic, relapsing–remitting disorder of the gastrointestinal tract, characterized by ulceration and consisting of two major forms: Crohn’s disease and ulcerative colitis [1, 2]. The main goal of IBD

treatment is to alleviate clinical symptoms and suppress intestinal inflammation but therapeutic agents are often associated with adverse effects and their lifelong use further impairs patient quality of life [3]. In recent years, colon-targeted drug delivery systems have attracted considerable attention in the treatment of IBD because they enable site-specific drug release and achieve high local drug concentrations at inflamed colonic tissues while minimizing systemic exposure [4]. This approach enables site-specific drug release by exploiting physiological variations along the gastrointestinal tract, particularly the relatively higher pH environment of the colon compared with the stomach and small intestine, which can be utilized for pH-responsive drug delivery systems [5–7]. Colon-targeted drug delivery systems need to be developed with considering the intestinal physiological factors, such as microbiota content, pH of digestive fluids, and transit time [8]. Protecting the drug from the gastric conditions and providing controlled drug release in the colon is one of the major challenges in colon-targeted drug delivery. Various strategies have been

developed to overcome these challenges, including prodrug approaches, polymer-coated drug delivery systems, time-dependent systems, osmotic-controlled systems, and pressure-controlled systems [8–11].

The oral and rectal administration have significant limitations for colon targeted drug delivery. Although conventional oral administration may lead to fast drug release and reduced drug concentration at the site of inflammation, oral-to-colon targeted delivery systems have been developed to enable site-specific drug release in the colonic region. Rectal administration is limited in its ability to reach the proximal regions of the colon, whereas oral administration faces challenges in achieving effective colonic drug delivery. Therefore, the development of novel drug delivery strategies is essential to overcome these challenges.

Various drug delivery systems, including nanoparticles, microparticles, hydrogels, tablets and nanofibers have been employed in the treatment of IBD [12–14]. Among these nanofibers have emerged as a promising drug delivery systems due to their high loading capacity, high encapsulation efficiency, ability to provide controlled drug release, ease of application, and cost-effectiveness [8, 11, 15, 16]. These characteristics enable controlled and site-specific drug release, making nanofibers promising platforms for oral-to-colon targeted drug delivery. In addition, nanofibers can also serve as scaffolds in hybrid delivery systems, further enhancing drug loading capacity and therapeutic performance. The electrospinning method is the most widely method for the preparation of nanofibers with high drug loading capacity and high surface area. This method uses high voltage to prepare fine fibers from polymer solutions using a high voltage, and the nanofibers can be tailored for specific purposes such as drug delivery and tissue engineering [17–19]. Among various polymers investigated for nanofiber-based systems, Eudragit polymers such as Eudragit S100 and Eudragit L100-55 have gained particular attention due to their pH-sensitive properties, making them suitable for colon-targeted drug delivery. Eudragit S100, a pH-sensitive polymer that is insoluble in acidic conditions, can significantly reduce drug release in the gastric conditions, facilitating targeted delivery to the colon [7, 20, 21]. Eudragit® L100-55, an enteric polymer that dissolves at pH values above 5.5, was combined with Eudragit® S100 to modulate the release profile and achieve a gradual pH-dependent drug release along the gastrointestinal tract, ultimately supporting colon-targeted delivery [19, 22, 23].

Polymeric nanofibers, particularly those composed Eudragit polymers such as Eudragit S100 and Eudragit L100-55, have been investigated for use in colon-targeted drug delivery systems. These polymers are suitable for the release of active pharmaceutical ingredients (APIs) in the colon due to the pH-sensitive properties. Despite advances

in novel strategies, conventional pharmacological treatment continues to represent a fundamental component of IBD management.

The conventional treatment of IBD involves corticosteroids, antibiotics, aminosalicylates, immunomodulators, and biological agents. Among these, corticosteroids have demonstrated notable efficacy in the management of moderate to severe IBD [24–26]. HC, a corticosteroid derivative, is widely used in the treatment IBD by reducing inflammation in the colon, primarily through the inhibition of cytokine production from T lymphocytes [27]. HC, a corticosteroid with well-established anti-inflammatory activity, has long been used in the management of IBD due to its ability to suppress inflammatory cytokine production [28]. HC has long been used in the treatment of inflammatory bowel disease due to its potent anti-inflammatory activity. In clinical practice, HC formulations such as rectal enemas, foams, and suppositories are commonly used to deliver the drug directly to inflamed colonic tissue, thereby reducing systemic corticosteroid exposure and associated adverse effects [27–30]. Therefore, although the approach builds upon established strategies, it contributes to the field by demonstrating the feasibility and potential advantages of HC-loaded Eudragit-based nanofiber systems for localized IBD therapy.

Previous studies have investigated corticosteroid-loaded electrospun nanofiber systems prepared with Eudragit polymers for intestinal or colon-targeted delivery. For example, Xu et al. (2013) developed budesonide-loaded core/shell nanofibers containing an Eudragit S100 shell for oral colon-specific drug delivery, while Turanlı et al. (2019) reported methylprednisolone-loaded delayed-release nanofibers prepared with Eudragit L100-55 and Eudragit S100 [31]. These studies demonstrate the potential of Eudragit-based electrospun nanofibers for site-specific corticosteroid delivery to the lower gastrointestinal tract. To the best of our knowledge, this is the first study to develop colon-targeted, HC-loaded nanofiber formulations for oral administration in IBD therapy. In this study, nanofibers were selected as the drug delivery system for oral use because they offer a high surface area and provide controlled drug release suitable for colon-targeted delivery. A blend of Eudragit S100 and Eudragit L100-55 was incorporated into the formulation to prevent drug release in the acidic gastric environment and to promote release under colonic pH conditions. Since inflammatory bowel disease may affect different regions of the intestine rather than a single localized site, the combination of Eudragit® S100 and Eudragit® L100-55 was used to provide a broader pH-dependent release profile along the intestinal tract, thereby supporting drug delivery to inflamed intestinal regions including the colon. This approach is expected to increase the local efficacy of HC in the intestine, thereby improving therapeutic outcomes

and reducing systemic side effects. The formulations were comprehensively evaluated through in vitro release studies, ex vivo permeability and mucoadhesion tests, and cell culture-based cytotoxicity assays. Overall, this work presents a promising strategy for the design of pH-sensitive, nanofiber-based oral drug delivery systems that may advance colon-targeted therapy of IBD and related gastrointestinal disorders.

Materials and methods

Materials

HC was kindly gifted by Gen İlaç (Türkiye). Eudragit® S 100 and Eudragit® L 100–55 were kindly supplied by Evonik Röhm GmbH (Darmstadt, Germany). N, N-dimethylformamide (DMF) and ethanol (EtOH) were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Germany). All other chemicals, reagents, and solvents were of analytical grade.

Preparation of Polymer Solutions for Electrospinning Process

Eudragit S100 and Eudragit L100-55, widely used pH-responsive polymers in colon-targeted drug delivery systems, were used to prepare the electrospinning solutions. Both HC-free and HC-loaded formulations were prepared using different ratios of Eudragit S100 and Eudragit L100-55. The polymers were dissolved in a solvent mixture consisting of EtOH and DMF at a ratio of 7:3 (v/v). DMF and EtOH were selected as the solvent system due to their ability to effectively dissolve the polymer components and produce homogeneous solutions suitable for stable electrospinning (NE300, Inovenso, Turkey).

The required amounts of polymers were gradually added to the solvent mixture and stirred using a magnetic stirrer until completely dissolved and a clear solution was obtained. For drug-loaded formulations, HC was added to the polymer solutions and mixed under continuous stirring to ensure uniform drug distribution within the solution. The

solutions were stirred until homogeneous electrospinning solutions were obtained. DMF and ethanol were selected as the solvent system due to their ability to dissolve the polymer components and form a homogeneous electrospinning solution suitable for stable nanofiber formation. The compositions and codes of the polymer solutions are presented in Table 1. The parameter ranges were selected based on literature reports and experimental trials, and the conditions that produced stable and continuous nanofibers are presented in Table 1.

Characterization of the Polymer Solutions

The viscosity of polymer solutions was determined at room temperature using a cone/plate rheometer (Brookfield DV-III, spindle CPE-41, USA), and the results were compared to evaluate their appropriateness for electrospinning. Surface tension was analyzed with an optical tensiometer (Attention Theta Lite, Biolin Scientific, Finland), and the values were obtained through the Young–Laplace equation based on the geometry of pendant drops at the needle tip [29]. The conductivity of the solutions was further assessed using a conductivity meter (Hanna Instruments HI 9033, USA), and the measurements were recorded in microsiemens per centimeter ($\mu\text{S}\cdot\text{cm}^{-1}$).

Electrospinning Process

Nanofiber formulations were prepared using the electrospinning process with a single nozzle equipment (Inovenso Ltd, NE300, Turkey). Polymer solutions were drawn into a 10 mL plastic syringe, and to prevent deformation of the fiber during the process, non-woven cotton was placed in the rotating cylinder. The electrospinning process was carried out at room temperature, with a duration of 1 h for each polymer solution to ensure consistency between formulations. The optimal process parameters for each polymer solution were determined to ensure continuous fiber production, and these parameters are presented in Table 1. After electrospinning, the nanofiber mats were kept under ambient conditions to allow solvent evaporation and minimize possible solvent residues.

Table 1 Codes, compositions and process parameters of polymer solutions for electrospinning process

Code of formulation	HC (%)	Eudragit S100 (%)	Eudragit L-100-55 (%)	Applied Voltage (kV)	Rotation speed (rpm)	Feed rate (mL/h)	Distance (cm)
E1	-	15	-	18	100	2.5	16
E2	-	-	15	22	100	2	12
E3	-	7.5	7.5	20	100	2.5	13
E4	1	15	-	18	100	2.5	16
E5	1	-	15	22	100	2	12
E6	1	7.5	7.5	20	100	2.5	13

Physicochemical Characterizations of Nanofiber Formulations

Thermal Analysis

The thermal behavior of the nanofiber formulations was evaluated by DSC using a DSC 60 instrument (Shimadzu, Japan). The samples were weighed accurately and placed in aluminum pans, which were then sealed before analysis. Thermal scans were performed over a temperature range of 30–300 °C under a nitrogen atmosphere to evaluate the thermal properties of the formulations and to investigate possible changes in the physical state of HC within the nanofiber matrix.

FT-IR Analysis

Possible molecular interactions within the nanofiber systems were investigated by FT-IR spectroscopy using a spectrometer equipped with an attenuated total reflectance accessory (Perkin Elmer Spectrum 400, USA). The spectra of the samples were recorded over an appropriate wavenumber range to identify the characteristic functional groups of the formulation components and to assess possible interactions between HC and the polymer matrix.

XRD Analysis

XRD analysis was performed to investigate possible changes in the crystallinity of hydrocortisone, the polymers, and the nanofiber formulations during the preparation process. The measurements were carried out using an XRD. The samples were placed on a sample holder and scanned over a 2θ range of 0–50° under ambient conditions. The obtained diffraction patterns were analyzed to evaluate the crystalline or amorphous nature of HC and the polymeric nanofiber formulations and to detect possible changes in crystallinity after electrospinning.

Proton Nuclear Magnetic Resonance (1 H-NMR) Analysis

¹H-NMR spectra of HC, Eudragit® S100, and the selected nanofiber formulation were recorded using a NMR. The samples were dissolved in deuterated solvent and transferred into NMR tubes for analysis. Tetramethylsilane (TMS) was used as an internal standard for chemical shift calibration. The spectra were obtained at room temperature under standard acquisition conditions. The analysis was conducted to confirm the structural integrity of HC and polymer components and to evaluate potential chemical interactions or structural changes occurring during the formulation process.

Morphological Analysis

The surface morphology of nanofiber formulations was examined using Scanning Electron Microscopy (SEM, QUANTA 400 F Field Emission Thermo Fisher Scientific, USA). The morphologies were observed at different magnifications (5000X, 10000X, 20000X). The diameters of the nanofibers were measured from SEM images using ImageJ software. For diameter measurement, 100 fibers were randomly selected and analyzed to ensure statistical reliability [32].

Mechanical Analysis

The tensile properties of the electrospun nanofibers were determined using a texture analyzer (Stable Micro Systems, TA.XT Plus Texture Analyzer, UK). For this analysis, fibers were cut into rectangular strips (3 cm × 1 cm), and six samples from each group were tested. Stress–strain curves were generated by the instrument's software, and these curves were used to calculate both the tensile strength (MPa) and the percentage elongation at break of the fibers [33].

Mucoadhesion Studies

Mucoadhesion studies were performed using a texture analyzer (TA.XT Plus, Stable Micro Systems, UK) to evaluate the adhesion of nanofiber formulations onto colon tissue. Fresh bovine colon tissues used in the ex vivo permeability experiments were obtained from a local slaughterhouse immediately after routine food-processing procedures. No animals were sacrificed specifically for this study. In this procedure, each formulation was fixed to the lower end of the probe with double-sided adhesive tape. The probe was brought into contact with the tissue under a constant force of 0.2 N for 60 s, at a test speed of 1 mm/s. From the resulting force–distance plots, the work of mucoadhesion was determined. The work of adhesion per unit area (mJ/cm²) was calculated as the area under the force–distance curve (AUC) divided by the probe contact area (πr^2), according to the formula ($\text{mJ/cm}^2 = \text{AUC}/(\pi r^2)$) [34].

Contact Angle Measurements

Contact angle measurements of nanofibers were carried out on an optical tensiometer (Attension-Teta Lite, Biolin Scientific, Finland). For contact angle measurements, a drop of liquid was placed on the nanofibers and the contact angle values of the nanofibers were calculated using the software of the device [35].

In Vitro Release Studies

In vitro release studies to determine the release rate of HC from nanofiber formulations were carried out using the USP apparatus I (basket) at 37 ± 0.5 °C and 500 rpm in a 500 mL dissolution medium under sink conditions. To simulate gastrointestinal conditions, release studies were carried out at pH 1.2, 6.8 and 7.4 buffers for 0–2, 2–6 and 6–24 h, respectively [36].

At each predetermined time points, 5 mL sample was collected and as an equal volume of the fresh buffer was added to the medium to maintain constant volume. The absorbance of the samples was measured using a ultraviolet (UV) spectrophotometer at 248 nm. Method validation was performed per ICH Q2 (R2) for linearity, accuracy, precision, specificity, LOD/LOQ, and robustness. Calibration curves were prepared using standard HC solutions within the appropriate concentration range, and the regression equation and correlation coefficient (R^2) were determined. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the response and the slope of the calibration curve. The method demonstrated adequate linearity and sensitivity in the media used for dissolution and permeability studies.

After completing the release studies, the release behavior of HC from nanofiber formulations was analyzed for conformity with the kinetic models. The evaluation was performed using DDSolver software, with model selection based on the adjusted R^2 (r^2_{adj}), Akaike Information Criterion (AIC), and Mean Square of the Centroid (MSC) values.

Ex-vivo Permeability Studies

Ex-vivo permeability studies were performed to assess the permeability of the formulations through bovine colon tissue using Franz diffusion cells at 37 °C and pH 7.4. HC-loaded nanofiber formulations were placed in the donor compartment, while pH 7.4 phosphate buffer was added to the receptor compartment of the Franz diffusion cell. Fresh bovine colon tissue was placed between the donor and receptor compartments, with the inner surface facing the donor. At predetermined time intervals, 2.5 mL samples were collected from the receptor phase, and an equal volume of fresh buffer was added to maintain the volume. The samples were analyzed using a UV spectrophotometer (UV-VIS-1800, Shimadzu, Japan, Shimadzu, Japan) at 248 nm, and the flux values for the nanofibers were calculated.

Cell Culture Studies

In inflammatory bowel disease (IBD), the integrity of the mucosal epithelial barrier is compromised, leading to the secretion of chemokines and proinflammatory cytokines by macrophages. Macrophages play an important role in the

host immune response during infection and disease progression. In patients with IBD, intestinal immune cells and macrophages secrete high levels of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) (Kim et al. 2015). RAW 264.7 macrophages have been extensively employed to study inflammatory responses and the modulation of pro-inflammatory cytokines such as TNF- α in drug delivery and biomaterial studies related to intestinal inflammation [16, 37]. Although inflammatory bowel disease involves complex interactions between epithelial cells and immune cells, macrophage-based models provide a well-established platform for evaluating anti-inflammatory activity at the cellular level. Therefore, the RAW 264.7 macrophage model was selected to assess the TNF- α -mediated inflammatory response of the developed nanofiber formulations. To evaluate the cytotoxicity and inflammatory activity of the nanofiber formulations, Western Blot Analysis and Real-Time Polymerase Chain Reaction (PCR) were performed on RAW 264.7 macrophage cells.

The RAW 264.7 macrophage cell line (RAW 264.7 murine macrophage cells (ATCC[®] TIB-71[™], American Type Culture Collection, Manassas, VA, USA) was cultured in 75 cm² flasks at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS (Fetal Bovine Serum-ATCC) and antibiotics (penicillin-streptomycin, ATCC) according to the manufacturer's protocols. Cell confluency was monitored using the software of the Juli Br device (Seoul, South Korea), and when the cell reached 70% confluence, they were subcultured with 3 ml of 1:3 Trypsin-EDTA solution per flask, once a week. For the application of the formulations to the cells; cells were transferred to 96-well plates as 1×10^4 per well.

Cytotoxicity Studies

The cytotoxicity of nanofibers and the polymers used in nanofiber production (Eudragit L100-S55, Eudragit S100, HC) was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The formulations and polymers were applied to 96-well plates, and the MTT test protocol was performed after 24 h of incubation. Since the MTT assay is a light-sensitive, the entire procedure was carried out in the dark. After incubation, the plates were mixed on a horizontal shaker, and their optical densities were analyzed at 540 nm wavelength in a microplate reader (SpectraMax i3 Multi-Mode Platform, Molecular Devices, USA). Cytotoxicity in sample-applied cells was expressed as a percentage (%) and calculated using the following formula:

$$\text{Cytotoxicity (\%)} = \left[\frac{1 - (Test - MinV)}{MaxV - MinV} \right] \times 100$$

Where:

- **MaxV** (Maximal Viability) corresponds to the positive control group (untreated cells), considered to be 100% viable.
- **MinV** (Minimal Viability) corresponds to the negative control group (treated with Triton-X), considered to be 0% viable [38].

Western Blot Analysis

Cell lysates were prepared by breaking open the cells, and the protein-containing fraction was separated from other cellular components. To lyse the cells or tissue, 100 μ L of RIPA buffer (containing 1% protease and phosphatase inhibitors) was added to the cell pellet. The mixture was kept on ice for 30 min, vortexing every 5 min. Sample buffer containing 10% mercaptoethanol was added to the protein samples in the appropriate amount, and the samples were heated at 90 °C for 5 min. A pre-cast 4–15% Tris-Glycine sodium dodecyl sulfate (SDS) gel was placed in the electrophoresis cassette, and the tank was filled with running buffer, with the inner pool being prepared first. A 10 μ L molecular weight standard was loaded into the first well, and 30 μ L of protein sample was loaded into the remaining wells. The samples were then separated by electrophoresis for approximately 1 h at a constant voltage of 100 V. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). To minimize non-specific binding, the membrane was incubated with a skim milk powder-TBST (Tris-buffered saline with 0.1% Tween-20) mixture for 1 h at room temperature. The membrane was then washed with TBS-T and blocked with blocking solution (Bio-Rad blocking buffer) for 1 h. For the detection of TNF- α , the primary antibody was diluted 1:1000 or 1:2000 in TBS-T. The membrane was incubated overnight at 4 °C with constant shaking in the antibody solution. After incubation, the membrane was washed 3 times with TBS-T for 5 min each. The membrane was then incubated for 1 h in a secondary antibody solution (anti-goat alkaline phosphatase-conjugate) diluted 1:10,000 (or 1:5,000) in TBS-T. Following this, the membrane was washed 3 times with TBS-T and twice with TBS for 10 min each, with gentle shaking. To visualize the protein bands, the membrane was treated with the BCIP/NBT color development solution (5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) (Sigma-Aldrich, Cat. No. B6404). The membrane was removed from the solution

once the bands became visible. The staining solution was discarded, and the membrane was washed with TBS for 10 min with shaking. The images were captured using an imaging device and analyzed with the Image Studio Imaging software (LI-COR Biosciences, Lincoln, NE, USA).

Determination of TNF- α Expression Levels by Real-Time PCR Method

In accordance with the Western blot application method, formulations were applied separately to RAW264.7 macrophage cells in the presence of 1 μ g/ml LPS at an IC₁₀ dose and incubated for 24 hours. Following this, RNA was isolated from cells. The quality of the RNA samples was determined with NanoDrop ND-1000 Spectrometer (NanoDrop Technologies, Wilmington, DE, USA). Only samples with a 260/280 absorbance ratio between 1.8–2.2 were included in the study. If the RNA quality was below this threshold, the isolation process was repeated. The unstable mRNA was reverse transcribed into stable cDNA, and the RNA expression levels of the target genes were evaluated using the PCR method. Random hexamers were used as a primers for cDNA synthesis to ensure comparable RNA amplification at each gene locus. If the cDNA quality was insufficient, the RNA stocks were used to repeat the cDNA extraction process. The differences in the expression levels of the TNF- α gene in the cells from which cDNAs were examined using the real-time quantitative PCR (qPCR) method using SYBR Green on the Light Cycler[®] 480 Real-Time PCR System (Roche Applied Science, Germany). For the qPCR method used to examine the changes in gene expression levels; two primers determined for TNF- α (Forward: 5'- CCCTCACA CTCAGATCATCTTCT- 3'; Reverse: 5' GCTACGACGTG GGCTACAG- 3')), SYBR Green dye that will fluoresce at the level associated with them, and a mixture called Master Mix containing Mg, dNTP and Buffer required for PCR were used. Plates containing 96 wells were used for each PCR reaction. The statistical significance of the differences observed in gene expression levels was determined based on the qPCR analysis results.

Statistical Analysis

Statistical analyses in the study were performed by Graphpad Prism[®] 12 software, using one-way and multi-way ANOVA to determine the difference between the means, and accepting the significance of the difference at the level of $p \leq 0.05$.

Results and Discussion

Characterization of the Polymer Solutions

The physicochemical properties of solutions directly affect the entire electrospinning process. The polymer solution intended for electrospinning was characterized by conductivity, surface tension, and viscosity analyses. These parameters are crucial in determining the optimum solution for the electrospinning process [39]. The results are presented in Table 2.

Various studies have investigated the effects of solutions with different conductivity values on the electrospinning process. Solutions with conductivities ranging from 0 to 2000 $\mu\text{S}/\text{cm}$ can be used for electrospinning. Notably, within the range of 0 to 150 $\mu\text{S}/\text{cm}$, increasing conductivity has been associated with improved continuity of the electrospinning process and a reduction in fiber diameter [40]. The results of the conductivity analysis in our study are given in Table 2. When examining the conductivities of the solutions used, it was observed that the addition of HC led to an increase in conductivity. Although the addition of HC may increase the conductivity of the polymer solution, which generally favors the formation of thinner fibers during electrospinning, other parameters such as solution viscosity, polymer concentration, and drug–polymer interactions can also influence the final fiber morphology. Drug loading may influence the physicochemical properties of electrospinning solutions, including conductivity, viscosity, and surface tension. Changes in solution composition can modify intermolecular interactions and charge density within the polymer solution, which may affect the electrospinning process and the morphology of the resulting nanofibers [41–43]. Variation in fiber diameter may result from the combined effect of these parameters rather than conductivity alone.

Polymer concentration exhibits a strong correlation with solution viscosity [44]. Viscosity values between 100 and 4000 mPa.s are considered suitable for electrospinning. It has been reported that solutions with viscosity values below 100 mPa.s tend to produce fibers with an increased frequency of beaded structures [45]. All viscosity values obtained in our study fall within this suitable range (Table 2) and are therefore appropriate electrospinning.

The surface tension of the polymer solution can directly affect the morphology and surface properties of the resulting fibers. It has been reported that surface tension is largely dependent on the solvent components used in the solution [46]. In our study, since both the polymers and the solvent system were kept constant, no significant differences were observed in the surface tension values (Table 2). In a study, the surface tensions of polymer solutions for methylprednisolone-loaded delayed-release nanofibers were reported to be in the range of 20.5–24.4 $\text{mN}\cdot\text{m}^{-1}$. Similarly, in our study, the surface tension values ranged from 26.36 to 27.93 $\text{mN}\cdot\text{m}^{-1}$ [31]. All measured surface tension values were within a range considered appropriate for electrospinning, enabling the successful production of nanofibers.

The physicochemical properties of the electrospinning solution may also be influenced by the structural differences between Eudragit S100 and Eudragit L100. These polymers differ in the ratio of methacrylic acid to methyl methacrylate groups, which can affect their ionization behavior and intermolecular interactions in solution. Eudragit L100 contains a higher proportion of carboxylic acid groups compared with Eudragit S100, which may increase the ionizable groups present in the solution and contribute to higher electrical conductivity. In addition, differences in polymer chain interactions and solution structure may influence viscosity and surface tension, which are key parameters governing electrospinning behavior and nanofiber formation.

Physicochemical Characterization of Nanofiber Formulations

Following the initial physicochemical characterization of all formulations, selected formulations exhibiting favorable properties were subjected to further permeability and biological studies.

Thermal Analysis of Formulations

DSC thermograms of HC, the polymers, and nanofibers are shown in Fig. 1. Upon examining the DSC thermograms, the melting peak of HC was observed at 226 °C. In

Table 2 Characterization of polymer solutions (Mean $\pm$ SD, $n=3$)

Formulation Code	Viscosity (cPs)	Surface Tension ($\text{mN}\cdot\text{m}^{-1}$)	Conductivity ($\mu\text{S}\cdot\text{cm}^{-1}$)
E1	3504 $\pm$ 555	27.67 $\pm$ 0.08	27.67 $\pm$ 0.08
E2	2538 $\pm$ 286	27.93 $\pm$ 0.09	26.54 $\pm$ 0.12
E3	3115 $\pm$ 76	27.89 $\pm$ 0.15	27.64 $\pm$ 0.21
E4	3418 $\pm$ 205	27.56 $\pm$ 0.08	36.54 $\pm$ 0.28
E5	3790 $\pm$ 53	29.93 $\pm$ 0.37	40.43 $\pm$ 0.05
E6	3035 $\pm$ 92	26.36 $\pm$ 0.09	39.36 $\pm$ 0.13

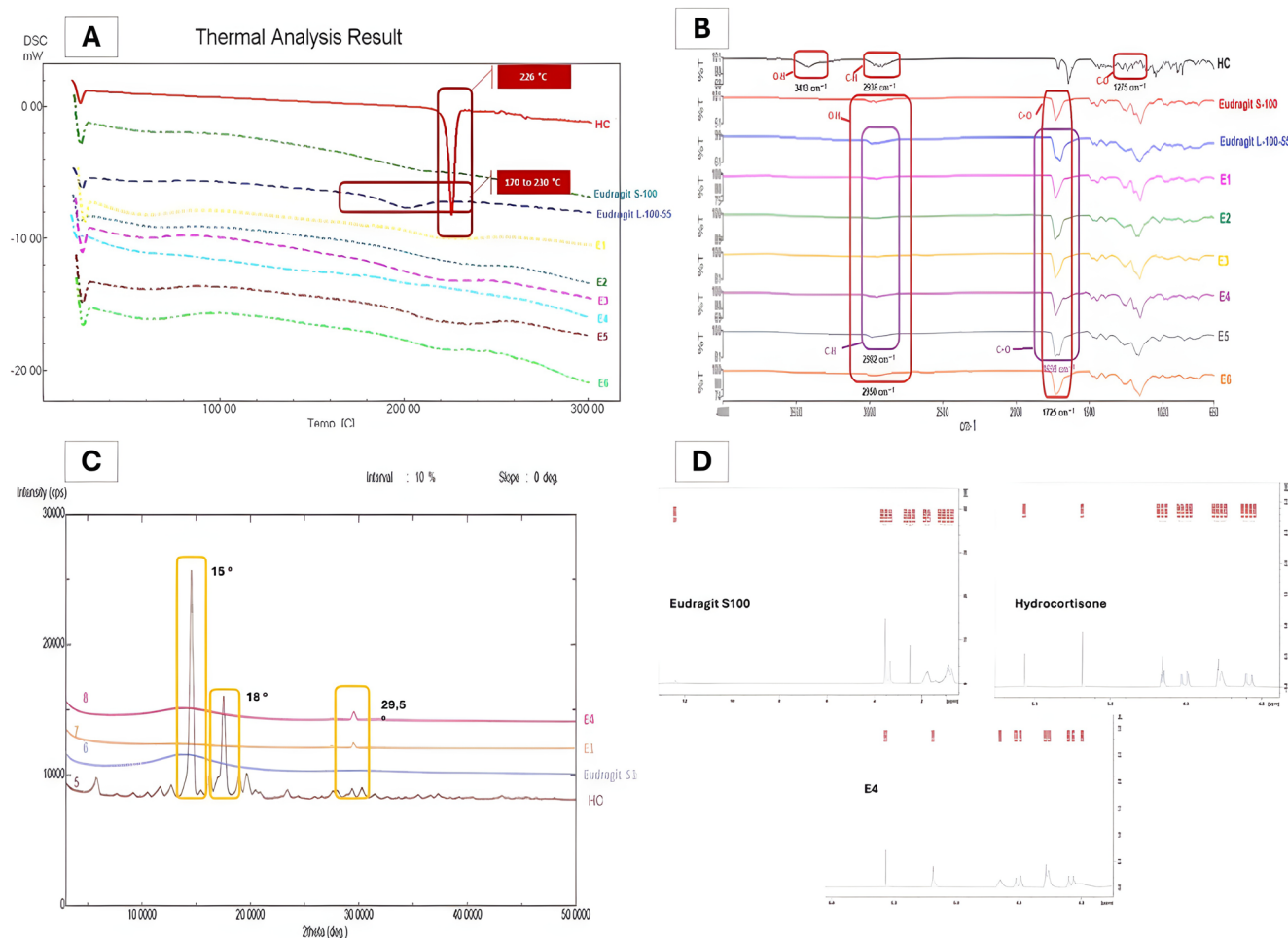


Fig. 1 a: DSC thermograms, b: FTIR spectra c: XRD patterns of HC, polymers and nanofiber formulations, d: ^1H -NMR spectra of Eudragit S-100, HC and E4 formulation

the literature, the melting peak of HC has been reported to be 226 °C and 224 °C [47, 48]. The melting peak of HC was observed in all formulations, indicating that the structure of HC remained unchanged during the electrospinning process. However, in nanofiber formulations, the HC peaks were not clearly visible due to the encapsulation of HC within the fibers, and its concentration was lower compared to the polymer. Eudragit[®] polymers exhibited broad thermal transitions without distinct melting peaks, which is consistent with their amorphous structure. Eudragit[®] L100-55 exhibited a broad and weak thermal transition without a distinct melting peak, which is consistent with the amorphous nature of the polymer. The absence of characteristic HC peaks in the nanofiber formulations may be attributed to the relatively low drug loading and the possible overlap of polymer absorption bands, which may mask the signals of the drug. In addition, HC may be molecularly dispersed within the polymer matrix after electrospinning, which can further reduce the detectability of its characteristic peaks.

Fourier-Transform Infrared Spectroscopy (FT-IR) Analysis

FT-IR spectra of HC, the polymers, and prepared nanofiber formulations are shown in Fig. 1. Specific peaks belonging to HC and Eudragit polymers are clearly observed in the spectrum. Upon examining the FT-IR spectra of the nanofibers, the characteristic peaks of HC are not visible in the nanofiber formulations. However, the polymers exhibit their characteristic peaks in the formulations. For Eudragit S100 and Eudragit L100-55, the following characteristic bands were observed: the C-H stretching band at 3000–2900 cm^{-1} , a strong esterified carbonyl group (C=O) stretching band at 1725 cm^{-1} , the methyl group stretching bands at 1437 and 1386 cm^{-1} , the acid C-O stretching band at 1247 cm^{-1} , and the ester C-O-C stretching bands at 1190 and 1147 cm^{-1} [49]. Characteristic bands corresponding to the Eudragit derivatives were observed in all formulations. For HC, the C-H stretching band was found at 2936 cm^{-1} , the O-H stretching band at 3413 cm^{-1} , and the C=O stretching band appeared in the range of 1600–1700 cm^{-1} [47].

X-ray Diffraction (XRD) Analysis

XRD analysis was conducted for the selected optimum nanofiber formulation to evaluate possible changes in crystallinity after electrospinning. XRD patterns of HC, polymers and nanofiber formulations are shown in Fig. 1. According to the XRD results, the peak belonging to the polymer was more clearly observed in the E4 formulation. A peak of HC was also observed in the E4 formulation. HC exhibited distinct peak points at diffraction angles (2θ) of 14.500° and 16.200° [47].

¹H-NMR Analysis

¹H-NMR analysis was performed to confirm the structural integrity of HC and the polymer components and to evaluate possible chemical interactions occurring during the formulation process. Analysis was carried out for the raw materials and the selected optimum nanofiber formulation. NMR is a technique used to help identify or confirm the structure of compounds. ¹H-NMR analysis is shown in Fig. 1. ¹H-NMR peaks between 5.5 and 5.0 ppm of HC are seen to be similar to the graph of the E4 formulation [50]. The ¹H-NMR spectrum of the E4 formulation showed characteristic proton signals corresponding to HC and Eudragit[®] S100, confirming that the structural integrity of the formulation components was preserved after the electrospinning process. The ¹H-NMR spectra showed that the characteristic proton signals of HC were preserved in the nanofiber formulation, indicating that the chemical structure of the drug remained after the electrospinning process.

Morphological Analysis

As shown in Fig. 2, the smooth and homogeneous appearance of nanofibers is a critical indicator of a successful electrospinning process. Such morphology enhances the application potential of nanofibers by increasing their structural integrity and functional effectiveness [51].

Fiber diameters in the nanometer range provides features such as an increase in specific surface area and high porosity, enabling better interaction of the formulation with colonic tissue. In the E4 formulation, HC addition increased the conductivity of the solution, which in turn reduced the fiber diameter. Although conductivity also increased in other formulations, their fiber diameters did not decrease. An increase in viscosity of polymer solutions causes an increase in the diameter of the fibers [52]. This effect could be attributed to changes in viscosity, surface tension, or polymer-specific interactions.

Fiber diameters tended to increase with HC loading (Fig. 3). The lowest fiber diameter among the formulations

containing HC was obtained in the E5 formulation. Bead formation was observed in the E2 and E5 formulations (Fig. 2). Fibers produced with Eudragit L 100–55 had the smallest diameter. According to the literature, when methanol or ethanol was used as the solvent in nanofibers produced with Eudragit[®] L 100–55, nanofibers could be obtained, but due to the high vapor pressure of ethanol and methanol, caused that the fibers with larger diameters [53]. In the literature, DMF was used in the preparation of polymer solutions. The formulations showed homogeneous appearance and cylindrical structure.

The diameter of electrospun nanofibers is strongly influenced by the physicochemical properties of the electrospinning solution, particularly viscosity, electrical conductivity, and surface tension. The structural differences between Eudragit S100 and Eudragit L100 may contribute to variations in these parameters. Eudragit L100 contains a higher proportion of methacrylic acid groups compared with Eudragit S100, which may increase the number of ionizable groups in the solution and consequently affect electrical conductivity. Increased conductivity can enhance the stretching of the electrospinning jet under the applied electric field, which may result in the formation of thinner nanofibers. In contrast, higher solution viscosity caused by stronger polymer chain entanglement may restrict jet stretching and lead to the formation of thicker fibers. In addition, HC loading may further influence these solution properties by modifying intermolecular interactions within the polymer–solvent system, which can contribute to the differences observed in nanofiber diameters among the formulations [41, 42].

Mechanical Analysis of Nanofiber Formulations

The results of the mechanical properties of the formulations are given in Fig. 4. Figure 4A shows the tensile strength values of the nanofiber formulations, while Fig. 4B presents the elongation at break results. Mechanical properties are one of the most important properties used to evaluate the performance of the formulations during application. Stress and strain studies were used to determine the tensile strength and elongation at break of the nanofibers. The lowest tensile strength was obtained in the E4 formulation; the highest elongation at break was found in the E4 formulation.

According to the mechanical properties of the formulations, the highest tensile strength was obtained in E3 and E6 formulations. Abdel-Rahman et al. developed vancomycin-loaded Eudragit-based nanofiber formulations. In this study, addition of the active substance to the formulations caused a decrease in the mechanical properties. In addition, the study showed that the preparation of Eudragit polymers as a mixture affected the mechanical properties [54]. In our study, a decrease in mechanical properties was observed only in E6 formulation compared

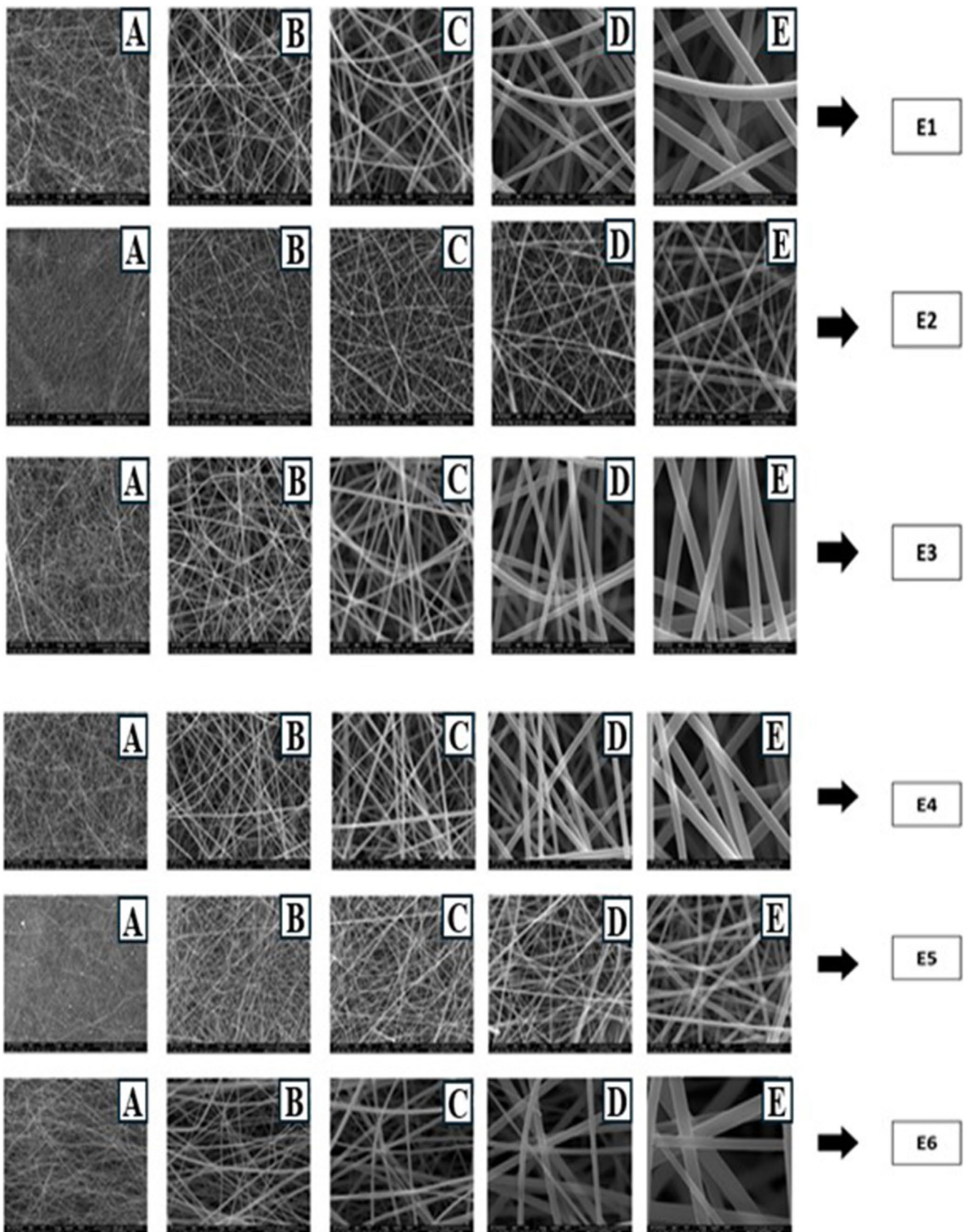


Fig. 2 SEM images of nanofiber formulations (Magnifications: **A**: 1000 $\times$, **B**: 5000 $\times$, **C**: 10000 $\times$, **D**: 20000 $\times$, **E**: 40000 $\times$)

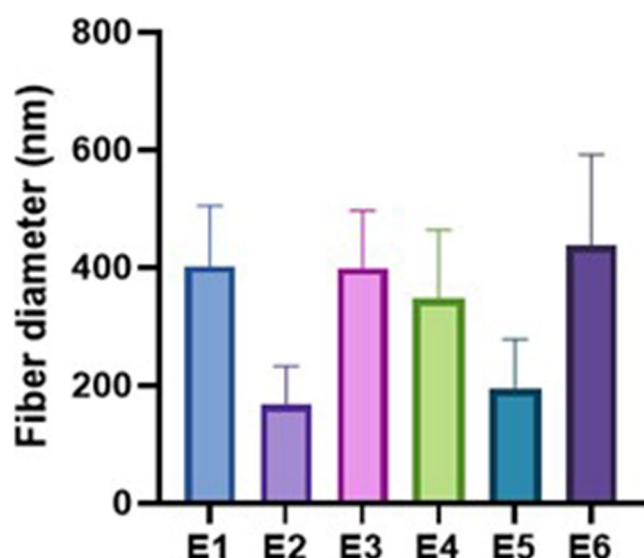


Fig. 3 Fiber diameters of nanofiber formulations (Mean $\pm$ SD, $n=100$)

to E3 with the loading of the active substance into the fibers. In addition, higher tensile strength was obtained by using both polymers as a mixture in our study.

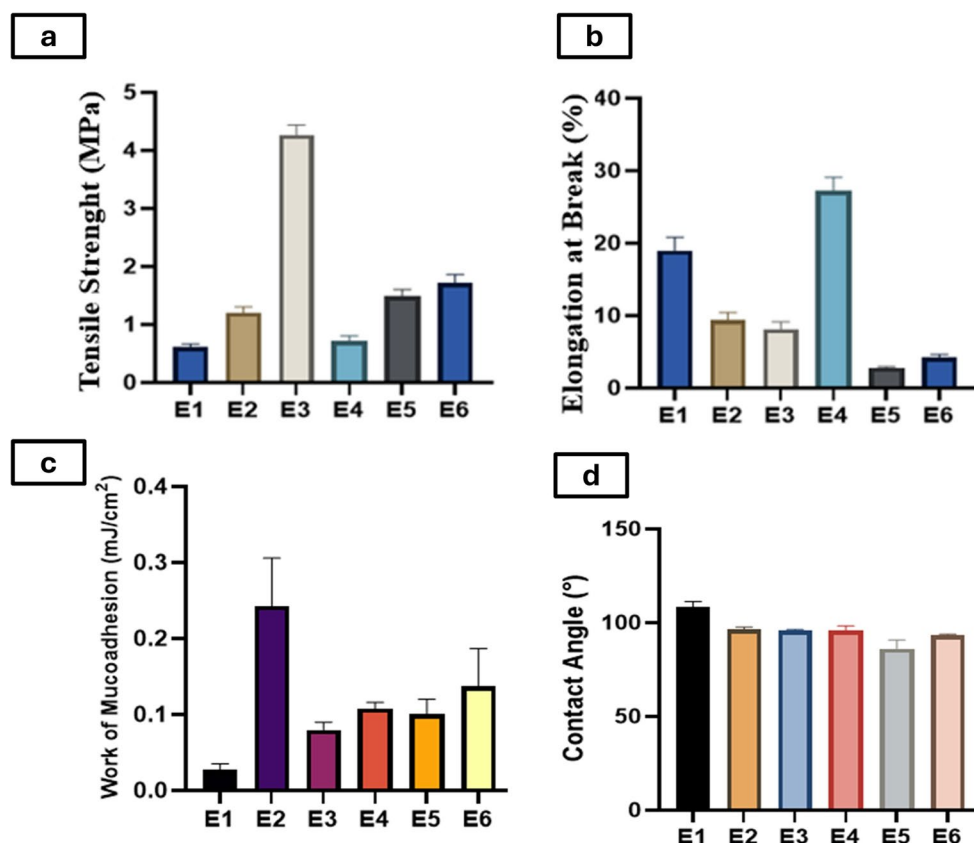
The mechanical properties of the nanofibers may be influenced by the structural differences between Eudragit S100 and Eudragit L100. Both polymers are anionic copolymers of methacrylic acid and methyl methacrylate; however, they

differ in the ratio of functional groups. Eudragit L100 contains a higher proportion of methacrylic acid groups (1:1 ratio), while Eudragit S100 has a lower content of acidic groups (1:2 ratio). The higher density of carboxylic groups in Eudragit L100 may promote stronger intermolecular interactions and hydrogen bonding within the polymer matrix, which can influence the mechanical behavior of the electrospun fibers. In addition, drug loading may further modify these polymer–polymer interactions by affecting the packing and organization of polymer chains within the nanofiber structure, thereby contributing to differences in mechanical properties among the formulations.

Mucoadhesion Studies

The mucoadhesion work results of the nanofiber formulations obtained on cow colon tissue using the Texture Analyzer device are presented in Fig. 4. Mucoadhesive systems increase the residence time in the colon [22]. Mucoadhesion primarily occurs through interactions such as hydrogen bonding, electrostatic interactions, and physical entanglement between polymer chains and mucin glycoproteins present in the mucus layer. In the present formulation, these interactions may contribute to the adhesion of the nanofiber matrix to the mucosal surface, potentially enhancing the residence time of the formulation at the target site.

Fig. 4 Mechanical properties of the nanofiber formulations: (a) tensile strength, (b) elongation at break, (c) work of mucoadhesion measured using bovine colon tissue, and (d) contact angle values. Data are expressed as mean $\pm$ SD ($n=3$)



The hydrophobic nature of the E1 formulation (Eudragit® S100) decreased wettability and tissue interaction, while its greater fiber diameter, associated with a lower specific surface area, led to reduced mucoadhesion. An increase in mucoadhesion work of E1 was observed with the addition of HC to the formulations (E4), similarly in the E3 formulation. However, in the E2 formulation, the addition of HC resulted in a decrease in mucoadhesion work. HC-loaded E4 and E6 formulations exhibited higher work of mucoadhesion compared with HC-free nanofiber formulations (E1 and E3). The highest mucoadhesion work among HC loaded formulations was obtained in the E6 formulation. The increase in mucoadhesion observed in HC-loaded formulations (E4 and E6) may be related to changes in the physicochemical properties of the nanofiber matrix after drug loading. HC may influence polymer–mucus interactions by modifying the surface characteristics of the fibers and the intermolecular interactions within the polymer network. Consequently, drug-loaded nanofibers may exhibit different mucoadhesive behavior compared with drug-free formulations. In addition, changes in fiber morphology and surface properties after drug incorporation may increase the effective contact area between the nanofibers and the mucus layer, thereby enhancing physical interactions. Mucoadhesion is largely governed by intermolecular interactions such as hydrogen bonding, electrostatic interactions, and polymer–mucin chain interpenetration, which are influenced by the physicochemical properties of the polymeric matrix [55].

Turanlı and Acartürk investigated the mucoadhesive properties of fibers formulated with Eudragit S100 and Eudragit RL100 using sheep colon tissue. They reported that although Eudragit RL100 is considered non-mucoadhesive, its positively charged structure facilitates strong electrostatic interactions with the negatively charged colonic mucosa, thereby enhancing residence time and enabling effective drug release [6]. The mucoadhesive properties of the polymers arise from their polyanionic nature, which allows the carboxylic acid (COOH) groups in their structure to form hydrogen bonds with the mucosal tissue. Eudragit S-100 and Eudragit L-100-55 showed mucoadhesive properties through their interactions with the mucosal tissue, as previously reported. In addition, the nanofibers structure provide interaction with the mucosa due to its large surface area and nanometer-scale pores [56].

Contact Angle Measurements

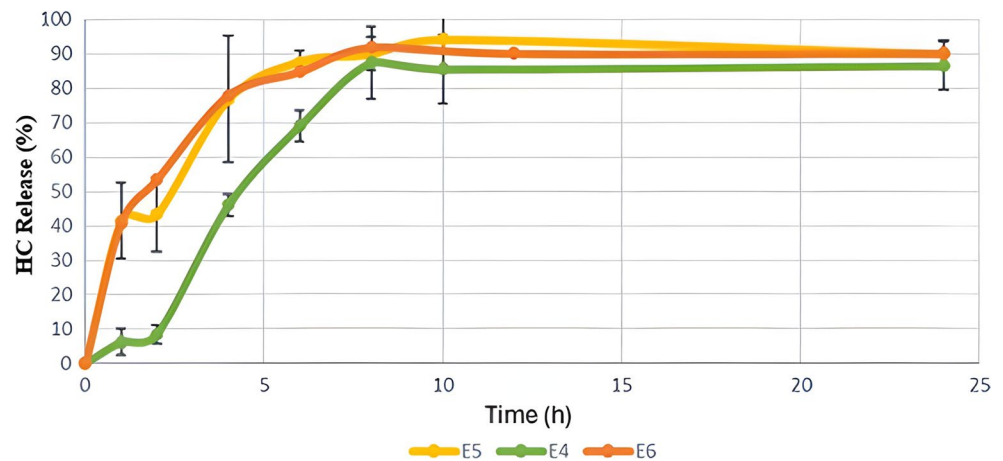
The contact angle of the fibers between 0–90° indicates good wettability, while the angle between 90–180° indicates inadequate wettability. Due to the hydrophobic structure of the Eudragit polymers used in our study, the contact angles were found to be higher than 90° except for the E5 formulation (Fig. 4). In vitro release of HC from the formulations occurs depending on the surface wettability. The highest contact

angle was found in the E1 formulation among the blank formulations; and in the E4 formulation among the HC-containing formulations. The addition of HC to the formulations decreased the contact angle in all formulations. Rade et al. stated that the contact angle of the metronidazole-loaded Eudragit S100 nanofibers showed high contact angles value due to the hydrophobic structure of the polymer [57]. The reduction in contact angle observed after HC loading may be attributed to changes in the surface characteristics of the nanofibers. The presence of HC within the polymer matrix may modify the surface chemistry and increase the wettability of the nanofiber structure. Increased wettability can facilitate the penetration of the dissolution medium into the nanofiber matrix, which may contribute to the drug release behavior observed in the in vitro release studies. Previous studies have also reported that increased wettability of electrospun nanofibers can enhance the interaction with aqueous media and influence drug release behavior [58].

In Vitro Release Studies

The in vitro 24-hour release profiles of the formulations obtained in USP App I are shown in Fig. 5. The E4 formulation released approximately 11.16% of HC at pH 1.2, i.e. in the first 2 h, and 74.33% at pH 6.8, i.e. in the 6th hour. The E5 formulation released approximately 45.2% of HC at pH 1.2 and 90.8% at pH 6.8. The E6 formulation released approximately 45.2% of HC at pH 1.2 and 83.96% at pH 6.8. These results indicate that the release from the E5 and E6 formulations occurred more rapidly at acidic pH compared to the E4 formulation. When the release results are examined, the E4 formulation provided the lowest release of HC in the stomach at pH 1.2, thus preventing the release of HC in the stomach and ensuring that it reached the intestinal area. Although nanofiber diameter can influence drug release behavior due to changes in surface area, drug release from electrospun nanofibers is governed by multiple factors, including polymer composition, drug distribution within the fibers, and polymer–drug interactions. Previous studies have shown that release kinetics from electrospun systems are not determined solely by fiber diameter but are also strongly affected by the physicochemical properties of the polymer matrix and the encapsulation characteristics of the drug [41, 58]. In the present study, although E2 and E4 exhibited relatively smaller fiber diameters, the E4 formulation showed lower HC release under acidic conditions. This suggests that matrix-related factors, such as polymer organization and drug entrapment within the Eudragit-based fibers, may play a more dominant role than fiber diameter alone. In contrast, the faster drug release observed in the E5 and E6 formulations may be associated with differences in matrix structure that facilitate more rapid drug diffusion under acidic conditions.

Fig. 5 In vitro release profiles of HC loaded E4, E5 and E6 formulations from dialysis membrane at 37 °C (Vertical bars represent the standard error, results are shown as mean $\pm$ standard error; $n=3$)



Turanlı and Acartürk developed budesonide-loaded nanofibers using Eudragit S-100 and Eudragit RL-100 in different ratios. They reported that the release rate of budesonide from the Eudragit S-100-based formulation was influenced by the pH of the medium. As the Eudragit RL100 polymer ratio increased, the pH sensitivity of the formulation decreased [6]. Similarly, in our study, the E6 formulation exhibited a faster release profile than the E4 formulation.

Karthikeyan et al. developed a composite fiber formulation containing aceclofenac and pantoprazole for colon-targeted delivery [23]. In these formulations, the release of pantoprazole from fibers prepared with Eudragit S 100 was investigated. Due to the characteristic structure of Eudragit S-100, which is insoluble in acidic solutions, only 6% of pantoprazole was released in the first 2 h at acidic pH. Akhgari et al. performed in vitro release in dissolution media with pH 1.2, 6.4, 6.8 and 7.4 to mimic various parts of the gastrointestinal tract [7]. Nanofiber formulations containing Eudragit RS100 and Eudragit S100 at different ratios were developed. The drug release rate in the acidic environment was found to be very slow in the formulations. In the formulations containing Eudragit S100, more resistance to drug release in the acidic environment was observed. In the formulation composed of entirely Eudragit S-100, drug release was less than 1% within 2 h. They stated that the presence of Eudragit S100 in the nanofiber structure even at low levels provided drug protection in the gastric environment [7]. Similarly, in our study, it was observed that approximately 11% of HC was released in the first 2 h at acidic pH in E4 formulation. S100-based nanofibers generally suppress release in the stomach, and release begins at intestinal/colon pH, consistent with the delayed, erosion-dependent profile we observed for E4. Sequential pH dissolution testing (pH 1.2–6.8–7.4) is widely used to evaluate the pH-dependent behavior of colon-targeted drug delivery systems and to simulate the major pH transitions along the gastrointestinal tract [7, 8]. However, the gastrointestinal environment is considerably more complex and may also involve enzymatic activity, microbiota-mediated degradation

processes, mechanical stress, and variable transit times. In addition, pathological conditions associated with inflammatory bowel disease, such as mucosal inflammation and intestinal dysbiosis, may alter local microenvironmental conditions and influence drug release behavior in vivo. Therefore, the current pH-based model represents a simplified approximation of the gastrointestinal environment, and further studies employing more biorelevant dissolution models or in vivo investigations may provide additional insight into the performance of the formulations.

Kinetic study of release profiles is performed to understand the release mechanisms of active substances and describe these processes with mathematical models. The kinetic modeling results of the formulations are given in Table 3. The release kinetics of the formulations fit the Hixon-Crowell, first-order and Weibull kinetics for E4, E5 and E6, respectively. Weibull kinetics shows compliance with the release profile from polymeric matrices. With the effect of Eudragites, their diameters first increased by swelling and then decreased by erosion over time, indicating the Hixson-Crowell model of release. This result shows that the polymer type may have changed the release mechanism. Ding et al. developed both monolithic and triaxial nanofiber formulations using Eudragit S100. When the release mechanism from the fibers was examined, release from both fiber formulations occurred by the erosion mechanism [19].

Ex-vivo Permeability Studies

The flux and permeability coefficients of nanofiber formulations, calculated using cow colon tissue, are presented in Table 4. In our study, the highest permeability value was found in the E6 formulation, at 80×10^{-3} cm/s. When the flux and permeability values were compared, the E5 formulation exhibited the lowest values; however, the difference was not statistically significant ($p>0.05$). Despite its more delayed and pH-dependent release, E4 exhibited similar ex vivo colonic permeability to E6, with no significant differences in

Table 3 Adjusted coefficient of determination (R^2_{adj}), Akaike information criterion (AIC), and model selection criterion (MSC) values of different mathematical models obtained from formulation data using DDSolver

Mathematical Models	Compliance Criteria	E4	E5	E6
Zero Order	r^2_{adj}	0.35623	0.69450	-0.86957
	AIC	72.5647	78.1256	78.3692
	MSC	-0.06545	-1.54461	-1.75650
First Order	r^2_{adj}	0.91667	0.95677	0.81328
	AIC	56.20775	48.77670	59.93825
	MSC	1.97916	2.12400	0.54737
Higuchi	r^2_{adj}	0.77764	0.59204	0.54683
	AIC	64.06048	66.73376	67.03157
	MSC	0.99757	0.12062	-0.33929
Korsmeyer-Peppas	r^2_{adj}	-0.00755	0.84462	0.88281
	AIC	76.91516	59.77814	56.97849
	MSC	-0.60925	0.74882	0.91734
Hixson-Crowell	r^2_{adj}	0.92992	0.72941	0.64943
	AIC	54.82274	63.44908	64.97793
	MSC	2.15229	0.28995	-0.08258
Weibull	r^2_{adj}	0.88055	0.93585	0.96943
	AIC	60.39739	54.24203	46.76964
	MSC	1.45546	1.56583	2.19344

Table 4 Flux and permeability coefficient parameters of formulations ($n=3$, mean $\pm$ SD)

Formulation Code	Flux ($\mu\text{g}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)	Permeability coefficient ($\text{cm}\cdot\text{h}^{-1}$)
E4	41.27 $\pm$ 19.92	0.066 $\pm$ 0.032
E5	36.02 $\pm$ 12.81	0.058 $\pm$ 0.020
E6	50.13 $\pm$ 13.79	0.080 $\pm$ 0.022

flux or permeability coefficient in ex vivo permeability studies ($p>0.05$). Christensen et al., showed that, in their studies on HC permeability through the skin using commercial preparations, the highest permeability value was 9.336×10^{-3} cm/s in a cream formulation [4]. Formulating HC as a nanofiber is expected to enhance its permeability by increasing surface area and porosity. Although E4 exhibited a slower and more

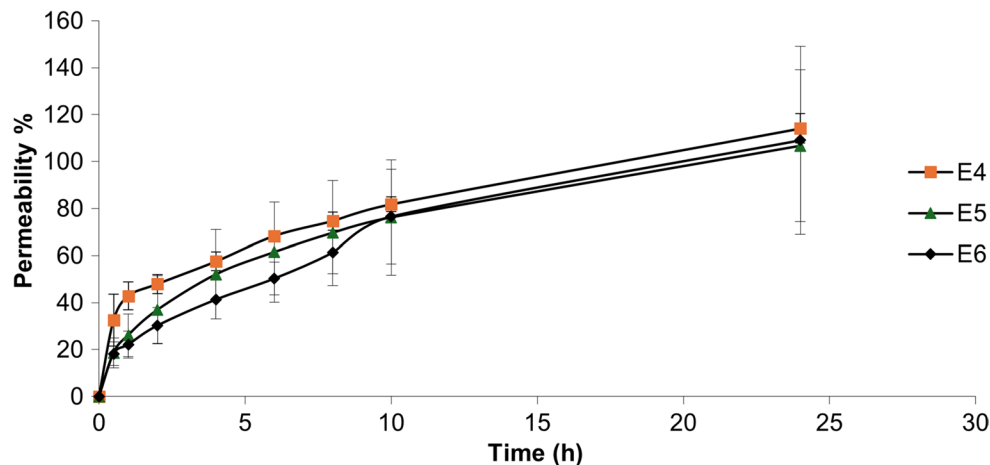
pH-dependent drug release profile compared with E6, similar permeability values may be attributed to differences in fiber morphology, polymer composition, and drug distribution within the nanofiber matrix. These factors may influence drug diffusion and interaction with the mucosal tissue, contributing to the observed permeability behavior. Rüzgar-Özemre et al. conducted a permeability study on rabbit intestinal tissue using sandwich fiber and nanoparticle-loaded sandwich nanofiber formulations, in which Eudragit S-100 was employed as both the bottom and top layers. They reported that the permeability of the formulations was higher, which they attributed to the increase surface area [59].

The cumulative permeation profiles of HC from nanofibers as a function of time are presented in Fig. 6. The permeation curves indicate a time-dependent increase in drug permeation for all formulations. Differences in the slopes of the permeation profiles suggest variations in permeation rates among the nanofiber formulations. These differences may be associated with variations in nanofiber structure, polymer composition, and drug distribution within the nanofiber matrix, which can influence drug diffusion through the membrane.

Cell Culture Studies

Cytotoxicity Studies

Cell viability data according to the applied concentration are shown in Fig. 7. Among the tested Eudragit-based nanofiber formulations, the highest cytotoxic effect was found in the E2 formulation. The lowest cytotoxicity among the HC-loaded formulations was found in the E6 formulation. In the E6 formulation, the use of both polymers in lower concentration may have caused a decrease in cytotoxicity. A decrease in cytotoxicity values was observed with the loading of HC. The difference in cytotoxicity between the formulations may have been due to the changes in the solvent residue, polymer ratio or the result of the electrospinning process. The % cell viability of the other formulations was found to be above 80%

Fig. 6 The cumulative permeation profiles of HC from nanofibers ($n=3$)

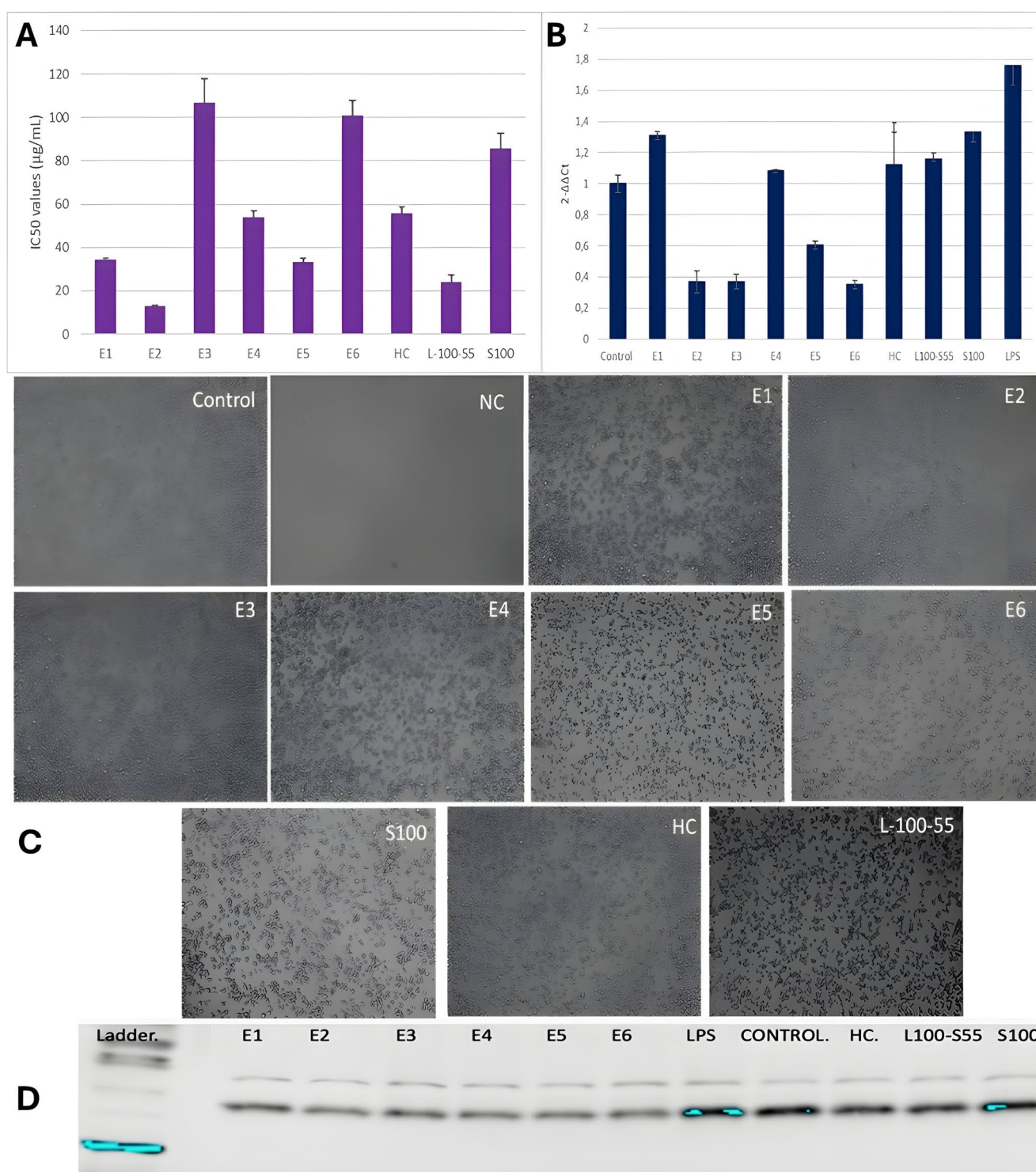


Fig. 7 Results of the cell culture studies on RAW 264.7 cell line **a**: IC50 values (µg/ml±SD) of formulations determined by MTT assay. **b**: Results of RT-PCR analysis on **c**: Morphology of RAW264.7 cells. (NC: Triton X-treated group, Control: Cells treated with only culture

medium, E1–E6: Nanofiber formulations, L-100-55, S100, and HC: Formulation contents.) **d**: Western blot analysis of TNF-α expressions in RAW264.7 cells

at the lowest dose of 1.93 $\mu\text{g/ml}$. The % cell viability value of the E4 formulation, which was determined as the optimum formulation, was found to be 88% at the same concentration. It was concluded that the formulations did not show a cytotoxic effect on the RAW 264.7 cell line. Formulation E2 exhibited notable cytotoxicity based on IC_{50} values, whereas the majority of formulations maintained RAW 264.7 cell viability above 80% at low concentrations. These findings indicate that the tested nanofiber formulations were well tolerated by RAW 264.7 cells, except at higher concentrations where cytotoxic effects became evident.

The images represent the morphology of RAW264.7 cells treated with IC_{10} concentrations of each formulation, which are sub-cytotoxic doses selected specifically for downstream applications such as Western blot and PCR analyses of TNF- α expression. At these low concentrations, no significant cytotoxic effects are expected. As anticipated, the NC group (Triton X-treated) exhibited complete cell lysis and detachment, confirming its strong cytotoxic effect, whereas the Control group maintained healthy, adherent morphology. Most formulations, including E2, E4, E5, E3, and E6, preserved normal cell shape and density, appearing morphologically similar to the Control group. Despite its low IC_{50} value, the E2 formulation did not induce visible morphological changes at the IC_{10} dose, indicating minimal structural impact at this level. These findings confirm that the IC_{10} concentrations used were suitable for investigating inflammatory responses without inducing overt cytotoxicity in RAW264.7 cells (Fig. 7).

Determination of TNF- α Expression Levels by Real-Time PCR (RT-PCR) Method

qPCR analysis of TNF- α mRNA expression in LPS-stimulated RAW264.7 cells showed that all nanofiber formulations effectively reduced TNF- α levels compared to HC alone, which is an important marker in IBD (Fig. 7). Among the tested formulations, E2 and E3 exhibited the greatest suppression of TNF- α mRNA expression (0.37 ± 0.07 and 0.37 ± 0.05 , respectively), consistent with the Western blot findings for protein levels. This was followed by E6 (0.35 ± 0.04), E5 (0.60 ± 0.03), and E1 (1.31 ± 0.03). The results for E2 and E1 were parallel to their protein expression profiles. No statistically significant difference was observed between E2 and E3 ($p > 0.05$). HC-loaded nanofiber formulations showed greater TNF- α reduction than blank polymers, confirming that HC incorporation enhances the anti-inflammatory potential. Cell-based assays demonstrated that the nanofiber formulations, particularly E2, E3 and E6 effectively reduced TNF- α expression at both the mRNA and protein levels while maintaining acceptable cell viability at IC_{10} concentrations.

Western Blot Analysis

Among the nanofiber formulations, the most significant reduction in TNF- α protein expression was observed in E2 (Fig. 7), which also demonstrated the highest efficacy in cytotoxicity findings (41% reduction). Other nanofiber formulations reduced TNF- α expression by 40% for E5, 39% for E4, 36% for E6, 34% for E3, and 20% for E1. The E4 formulation showed a particularly effective reduction in TNF- α despite its moderate cytotoxicity profile. As expected, HC-loaded formulations induced a greater decrease in TNF- α expression compared to their blank counterparts, indicating an anti-inflammatory contribution from the active compound. No significant difference was observed between the individual formulation components (L100-S55, S100, and HC alone). Overall, the RT-PCR results paralleled the Western blot data, highlighting E2, E3, and E6 as the most effective in downregulating TNF- α expression at both the gene and protein levels.

Conclusion

Nanofiber drug delivery systems enabling pH-sensitive drug release were successfully prepared by electrospinning using Eudragit polymers. Comprehensive physicochemical and morphological analyses confirmed the formation of uniform nanofibrous structures and indicated that HC was well dispersed within the polymer matrix without significant drug-polymer interactions. Drug release studies demonstrated that polymer type plays a critical role in determining the release behavior, with the electrospun nanofibers exhibiting a release mechanism involving swelling, diffusion, and erosion.

Among the tested formulations, the E4 formulation prepared with Eudragit S100 minimized HC release under gastric conditions while allowing substantial release in the intestinal environment, supporting its delayed colon-targeted delivery profile. In vitro studies demonstrated that the formulations were well tolerated by RAW 264.7 cells and reduced LPS-induced TNF- α expression at both mRNA and protein levels, indicating potential anti-inflammatory relevance for localized colonic delivery. The findings demonstrate that the polymer composition strongly influenced the physicochemical, mechanical, mucoadhesive, and release characteristics of the nanofiber systems. The physicochemical, ex vivo, and cell-based findings suggest that electrospun Eudragit nanofibers can improve the local delivery potential of HC by integrating pH-dependent release, mucosal interaction, and anti-inflammatory activity.

Overall, these findings suggest that HC-loaded Eudragit nanofibers represent a promising platform for colon-targeted drug delivery and warrant further evaluation in in vivo models.

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Data Availability All data supporting the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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