

Article

Cyclodextrin Polymer Complexation Improves the Tolerability of Parenteral Oestradiol

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Abstract

Background: Menopause is characterised by a decline in oestrogen levels, leading to physical and psychological symptoms that significantly affect quality of life. Current parenteral oestradiol ester therapies, while effective, are often associated with side effects due to their oil-based formulations, including injection-site reactions and immune responses. **Methods:** In this study, we developed a water-soluble, polyethylene glycol cross-linked β -cyclodextrin (PEG- β -CD) polymer-based system for parenteral oestradiol delivery and evaluated its biocompatibility, solubility enhancement, immune compatibility, and pharmacokinetics. **Results:** Cytotoxicity assays using NIH-3T3 fibroblasts and RAW 264.7 macrophages showed minimal toxicity up to 10% (*w/w*). Phase-solubility studies demonstrated a significant increase in oestradiol solubility with the PEG- β -CD polymer, surpassing that of β -cyclodextrin or PEG alone. Dynamic light scattering and FTIR analyses confirmed successful complex formation, with submicron particles averaging 271 nm and physical incorporation of oestradiol into the polymer matrix. Macrophage activation assays and RT-qPCR analyses indicated an absence of immunogenic responses or pro-inflammatory cytokine induction. In vivo toxicity testing in *Galleria mellonella* larvae confirmed safety, while pharmacokinetic studies in Wistar rats revealed rapid initial absorption followed by stable, low-level serum concentrations comparable to those of commercially used oestradiol esters. **Conclusions:** These findings indicate that the PEG- β -CD polymer-oestradiol complex provides a safe, water-based alternative to traditional oil-based injections, with the potential to reduce side effects and improve patient compliance in postmenopausal hormone therapy.

Keywords: oestradiol; cyclodextrin polymer; oestradiol formulation; menopause



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1. Introduction

Menopause is the cessation of menstruation for a minimum of twelve months, resulting from a decrease in oestrogen levels, and it is not associated with any abnormal disease. On average, women enter this phase of life at around 51 years of age. As life expectancy continues to increase, women may spend up to one-third of their lives in the post-menopausal phase [1]. Therefore, addressing the onset of symptoms such as hot flashes, flushing, night sweats, irritability, mood and sleep disturbances, fatigue, decreased libido, dyspareunia, and atrophic vaginitis is becoming an increasingly significant issue [2].

The symptoms experienced during menopause and post-menopause result from shifts in hormonal balance. Specifically, the ratio of oestrogen and progesterone is disrupted due to a reduction in oestrogen levels. These hormonal fluctuations can also contribute to an increased risk of cardiovascular disease and osteoporosis [3,4]. These periods can be burdensome on women not only physically but also mentally, leading to the availability of various therapeutic options, including hormone replacement therapy. It is crucial to personalise hormone therapy for each individual to mitigate the risk of cancer development associated with the treatment [5].

Nowadays, parenteral administration is often chosen for most therapies because of the low absolute oral bioavailability of endogenous drugs [6]. Intramuscular or transdermal administration of oestrogen, in contrast to oral administration, is associated with a lower risk of deep vein thrombosis, gall bladder disease, osteoporosis and stroke [7]. Administration of uncontrolled high levels of oestrogen in the body increases the risk of cancer [8], so modified derivatives are used in most cases.

Oestradiol esters constitute a cornerstone of contemporary oestrogen replacement therapy. Among these, oestradiol benzoate, oestradiol cypionate, and oestradiol valerate are the most widely used formulations [1,9]. Although intramuscular (IM) injection remains the primary route of administration, subcutaneous (SC) delivery is increasingly investigated in research and has demonstrated comparable pharmacokinetic characteristics [10,11]. Commercially available oestradiol esters are formulated as oil-based injections designed to produce a depot effect, enabling sustained release of oestradiol and minimizing abrupt fluctuations in serum hormone concentrations. This controlled delivery is intended to improve hormonal stability and reduce the theoretical risk associated with supraphysiological oestradiol peaks, including oestrogen-sensitive tumour development [8]. Despite their therapeutic efficacy, patient adherence to injectable oestradiol ester formulations remains suboptimal. This is largely attributable to injection-site complications associated with oil-based vehicles, such as localised pain, inflammation, sterile abscess formation, and, in some cases, hypersensitivity or immune-mediated reactions. Such reactions are largely attributed to oil-based vehicles (e.g., castor oil, sesame oil) rather than oestradiol itself. Delayed hypersensitivity reactions and immune-mediated responses have also been described [7,11,12]. These adverse effects represent a significant limitation of long-term parenteral oestrogen therapy and have prompted ongoing investigation into alternative delivery systems [7]. The most effective therapy would be to develop a formulation that delivers the active ingredient in a controlled way and does not cause irritation. Cyclodextrin molecules increase the solubility of oestradiol via inclusion complexes supported by hydrophobic interactions, van der Waals forces, and hydrogen bonding together [13,14] and control the release of the active substance [14].

Covalently linked cyclodextrin-containing macromolecular drug delivery systems have been investigated since the 1980s. Several types of these macromolecular systems can be distinguished based on their structure, such as linear polymers containing pendant cyclodextrin (CD) moieties, cross-linked cyclodextrin polymers or polyrotaxanes [14]. Modified CDs (α -, β - and γ -), in addition to their existing properties, offer new possibilities

to native CD molecules, such as gel formation and prolonged and smart drug delivery, i.e., they can change their properties in response to environmental influences [14].

Extensive literature exists on cyclodextrin monomers used in oestrogen therapy [13]. Polymers are also used to remove hormone contaminants because they can effectively bind them [15,16]. However, research also shows that various cyclodextrin polymers have been used as carriers or modified reservoirs for hormone replacement [17–19]. One high-potential variant that has been synthesised recently is polyethylene glycol cross-linked cyclodextrin polymer (PEG- β -CD polymer). This new polymer did not exhibit any cell-damaging effects at concentrations up to 10 *w/w*%. Complexation studies have shown that the polymer is effective in increasing the solubility of poorly soluble drugs in water (curcumin) and enhancing their penetration through the intestinal epithelium. The complexation mechanism occurs via inclusion of the drug in β CD units in a 1:1 molar ratio while the remaining important amount of the drug is entrapped in the free hydrodynamic volume of the water-soluble cross-linked polymer. The chemico-physical characteristics of the polymer make it suitable for the preparation of reservoir formulations for both parental and oral administration [20].

The aim of this study is to develop a PEG- β -cyclodextrin (PEG- β -CD) polymer-based, parenterally applicable therapeutic delivery system for oestradiol supplementation and to evaluate its performance *in vitro* and *in vivo*. This system is expected to enhance postmenopausal hormone therapy by providing a water-based alternative to traditional oil-based oestradiol ester formulations and reduce associated side effects.

2. Materials and Methods

2.1. Materials

The cross-linked β -cyclodextrin with polyethylene glycol diglycidyl ether (PEG- β -CD polymer) was synthesised at Cranfield University as previously described [19,20]. The beta-cyclodextrin (β CD) was purchased from Cyclolab Ltd. (Budapest, Hungary). PEG 400, β -oestradiol and other reagents were from Sigma-Aldrich Ltd. (Budapest, Hungary).

2.2. Cytotoxicity Test by MTT

The cytotoxicity of PEG- β -CD polymer was assessed using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. NIH-3T3 (mouse fibroblast) and RAW 264.7 (mouse macrophage) cell lines were sourced from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK) and are suitable for characterizing the immune system [21–25] and the parenteral environment [26–28] in the case of our preparation. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L D-glucose, 0.584 g/L L-glutamine and phenol red (Sigma-Aldrich), supplemented with 3.7 g/L NaHCO₃ (Sigma-Aldrich), 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 1% non-essential amino acids solution (Lonza, Basel, Switzerland), 100 IU/mL penicillin, and 100 μ g/mL streptomycin solution (Lonza) maintained at 37 °C in a 5% CO₂ incubator. For the RAW 264.7 cells, passages were kept between 5 and 15, while for NIH-3T3 cells, the passage number was maintained below 10. A total of 20,000 cells per well were seeded into 96-well plates. After 3 days, the medium was removed, and the cells were treated with various concentrations of cyclodextrin polymer (0.01%, 0.1%, 1.0%, 5.0%, and 10.0%) in DMEM for 24 h at 37 °C. The samples were then removed, washed with Hank's solution, and 0.5 mg/mL MTT in phosphate-buffered solution was added to each well. The plates were incubated for 2 h before the MTT solution was discarded, and 0.1 mL of isopropanol mixed with 1 M hydrochloric acid (25:1) was added to each well to dissolve the resulting formazan crystals. Formazan absorbance was measured at 570 nm, with background correction at 690 nm, using

a Thermo-Fisher Multiskan Go microplate reader (Thermo-Fisher, Waltham, MA, USA). The survival was compared to the control group.

2.3. Phase-Solubility Test

The phase-solubility test was performed by the following process [20]. Excessed oestradiol powder was added to each vial; these vials contain 1 mL water (pH 7.00) solution with an increasing amount (0.05; 0.1; 0.5; 1.0 $w/w\%$) of PEG- β -CD polymer, β CD, PEG 400 or β CD:PEG 400 1:1 ($w:w$) mixture. The vials were vortexed for 30 s to achieve well-mixed dispersions. They were rotated at room temperature at 50 rpm and protected from light. After 24 h, each vial was centrifuged at 4500 rpm for 20 min and the supernatant was filtered by 0.22 μ m polyether-sulfone membrane. A UV spectrophotometer (Shimadzu UV-1900, Shimadzu Corporation, Kyoto, Japan) was used to determine the oestradiol content of the supernatant (430 nm), after breakdown of the complex with a 50 $v/v\%$ ethanol solution. The phase-solubility profiles of oestradiol were achieved by plotting the solubility of oestradiol versus the $w/w\%$ concentration of the solubilizing agents (CD, PEG).

2.4. Preparation of Solid, Sterile Powder for Injection

A 10 $w/w\%$ polymer solution was prepared in purified water, and an excess amount of oestradiol was dispersed in it and then stirred at 50 rpm for 24 h to reach equilibrium. The suspension was centrifuged at 4500 rpm for 20 min, and the supernatant was filtered by 0.22 μ m membrane under the laminar box to separate and to reach sterilization. After that the clear supernatant was separated and lyophilised using a ScanVac CoolSafe freeze dryer (Labogene, Allerød, Denmark). The lyophilised product was then stored at $-20\text{ }^{\circ}\text{C}$ until further experimentation. The sterilised lyophilised sample was dissolved in sterile water for further tests.

The oestradiol content of complex was measured by HPLC. The 5 mg samples were dissolved in mobile phase. The solutions obtained were analyzed using an HPLC system (Merck-Hitachi ELITE with photodiode array detector (DAD), Hitachi High-Technologies Corporation, Tokyo, Japan). The column was an Agilent HC-C18(2) (150 $\times$ 4.6 mm), kept at $40\text{ }^{\circ}\text{C}$, and the DAD was set at 278 nm. The mobile phase was 2% acetic acid solution and methanol (3:7), and 1.0 mL flow rate was used. The analyses were performed with EZChrom Elite software TM (Hitachi, Tokyo, Japan), which was also used for collecting and processing the data. The injection volume of the samples was 10 μ L.

2.5. Dynamic Light Scattering Measurement (DLS)

Malvern Nano-ZS Zetasizer (Zetasizer Ver. 7.13, Malvern Instruments, Malvern, UK) was used to determine the average particle size of the polymer and complex. The sample and native polymer were dissolved to reach 0.5% concentrate solution in purified water. The results were plotted as a function of occurrence and measured particle size.

2.6. Fourier-Transform Infrared Spectroscopy (FTIR)

The infrared spectra of the pure component, physical mixture, and complex were obtained by using an JASCO FT/IR-4600 type (ABL&E-JASCO, Budapest, Hungary) apparatus coupled with a Zn/Se ATR PRO ONE Single-Reflection ATR (Attenuated Total Reflectance) accessory. Each sample was directly placed on an ethanol-water-cleaned crystal of the equipment; the scanning was run in the wavelength range of 500–4000 cm^{-1} , and the spectra were collected from 32 scans to obtain smooth spectra, at the spectral resolution of 1 cm^{-1} . Corrections of environmental CO_2 , H_2O and apparatus ATR used the software's built-in method.

2.7. Macrophage Activation

RAW 264.7 macrophages (2×10^5 cells/plate) were seeded on a 6-well plate. After 24 h, the cells were treated with medium, Lipopolysaccharide (LPS) and PEG- β -CD polymer-oestradiol complex for 24 h. The cells were then washed 2 times with PBS solution, then 2 μ L MHC-II labelling antibody was added in each well and incubated at room temperature for 1 h. The cells were suspended with PBS solution, and the washing cycle was repeated 2 times with ice-cold PBS to wash off the excess antibody. Flow cytometry measurement was used to detect the changes in cell membrane proteins in the following settings. The fluorescence intensity of the 1×10^4 cells was measured by a Guava Easy Cyte 6HT-2L flow cytometer (Merck Ltd., Darmstadt, Germany) using green (525/30 nm) channel. The mean green fluorescence values of the viable cells were evaluated. The effects of oestradiol-cyclodextrin polymer complex were compared to the untreated control and LPS treated in three parallel experiments.

Macrophage activation was also confirmed after the antibody labelling by Fluorescence Microscopy. In that case the cells were placed on glass coverslips to grow. After the MHC-II labelling process, the cells were fixed with a 3.7% paraformaldehyde solution for 15 min at room temperature. Then, cells were washed, and cell nuclei were labelled with 283 nM DAPI solution (4',6-diamidino-2-phenylindole) for 10 min at room temperature. Fluorescence microscopy investigations were conducted with a Zeiss Axioscope A1 (Carl Zeiss AG, Jena, Germany) fluorescence microscope. The following filter sets were used in the microscopy investigations: blue (DAPI): excitation G 365 nm, emission BP 445/50 nm; green (fluorescein): excitation BP 470/40 nm, emission BP 525/50 nm.

2.8. Inflammatory Factor Activation by RT-qPCR

RAW 264.7 macrophages (2×10^5 cells/plate) were seeded on a 6-well plate. After 1 day, they were treated with medium, LPS, IFN- γ or PEG- β -CD polymer-oestradiol complex. After 24 h, the seeded cells of total RNA were extracted using 1.00 mL Trizol reagent. Total RNA was prepared by phenol-chloroform extraction. RNA from each sample (2000 ng) was reverse transcribed to cDNA using a Tetra cDNA synthesis kit (Bioline, London, UK) in a final volume of 20 μ L. First-strand cDNA was amplified using gene-specific primers (Table 1). mRNA levels of TNF- α , IL-1 α , IL-1 β , IL-6, IL-10, GAPDH have been assessed using iQTM SYBR[®] Green Supermix (BIO-RAD, Hercules, CA, USA). Reactions were conducted according to the manufacturer's protocol using a MyiQ2 two-colour real-time PCR detection system (BIO-RAD, USA). All real-time amplifications were measured in triplicates. Results were evaluated with BIO-RAD iQ5 (BIO-RAD, USA) software, and changes in mRNA levels were calculated using the 240-Ct method.

Table 1. Nucleotide sequences of primers used in real-time qRT-PCR.

Gene	Forward (5'–3')	Reverse (5'–3')
TNF- α	AGCAAACCACCAAGTGGAGGA	GCTGGCACCAGTGTGGTTGT
IL-1 α	TTGGTTAAATGACCTGCAACA	GAGCGCTCACGAACAGTTG
IL-1 β	AGTTGACGGACCCCAAAAG	AGCTGGATGCTCTCATCAGG
IL-6	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA
IL-10	CAGAGCCACATGCTCCTAGA	TGTCCAGCTGGTCCTTTGTT
GAPDH (housekeeping gene)	AGGTCATCCCAGAGCTGAACG	CACCCTGTTGCTGTAGCCGTAT

2.9. *G. mellonella* Larvae Survivability Test

G. mellonella larvae survivability test was carried out as an in vivo toxicity test before the in vivo pharmacokinetic test to make sure the products are safe to use. Larvae of the sixth developmental stage of *G. mellonella* were obtained from Bugs World Inc. (Budapest, Hungary). Larvae were kept at 10 °C and in a dark environment prior to use. Larvae size

was between 2 and 3 cm and they showed no sign of melanisation. For each treatment, 20 healthy larvae were placed in sterile vented Petri dishes. The formulation was diluted with water for injection and 20 µL of each sample was injected into the *G. mellonella* haemocoel through the last pro-leg using a 29 G needle. The injected larvae were incubated at 30 °C for 48 h in a dark environment. For the assessment of larval viability, larvae were gently probed with a blunt-ended needle, and if no response was observed, the larvae were deemed dead. Viability was observed at 15, 24, 39, and 48 h.

2.10. In Vivo Pharmacokinetic Test

The pH and osmolality (mOsmol/L) of the preparation were also checked before the animals were treated, in addition to the active substance content. The sterile lyophilised sample was dissolved in sterile water for injection to get 20 w/w% solution. The pH of the sample was measured by Mettler Toledo Seven Easy desktop pH meter (Mettler Toledo, Greifensee, Switzerland). The osmolality of the formulation was measured by OSMOMAT 070 vapor pressure osmometer (GONOTEC GmbH, Berlin, Germany).

The pharmacokinetic study was carried out in compliance with the regulations set by the Hungarian Scientific Ethical Committee on Animal Experimentation (Approval number: 7/2023/DEMÁB, approval date: 14 December 2023). We used GPower (<http://www.gpower.hhu.de>, accessed on 2 May 2023) software to estimate the number of subjects involved in the experiments ($\alpha = 0.05$; assumed effect size: 1; calculated power: 0.9548089). A total of 24 male Wistar rats, aged 12–36 weeks and weighing 200–450 g, were included in the experiment as a well-established and widely accepted species for pharmacokinetic and hormone-related studies. The animals were housed under controlled conditions at 22 ± 2 °C with a relative humidity of $50 \pm 10\%$, and a 12 h light/dark cycle, while the environment was enriching. The rats had unrestricted access to water, and food was provided ad libitum. Due to the duration of the experiment (1 month), a humane endpoint was established to reduce animal suffering, whereby if an animal lost $\geq 20\%$ of its body weight, did not move for ≤ 24 h, or lacked a startle reflex, it was removed from the experiment and euthanised. For the experiment, the rats were divided into two main groups: control (n = 12) and treated (n = 12). Each of these groups were further divided into two subgroups based on the route of injection (subgroup A: subcutaneous (n = 6), subgroup B: intramuscular (n = 6)). Simple random sampling was used to choose the animal into group. In all cases, the animals were anaesthetised with an intraperitoneal injection of ketamine/xylazine (50/10 mg/kg body weight). The rats were then administered 200 µL of a 0.9% NaCl solution as a control or 200 µL of a 20 w/w% oestradiol polymer complex solution as the test treatment, either subcutaneously or intramuscularly. Blood samples (200 µL) were collected from the tail vein at intervals of 1 day, 3 days, 1 week, 2 weeks, 3 weeks, and 1 month post-injection. The collected samples were processed by centrifugation (3500 rpm for 10 min) to remove cellular components, and the resulting serum was stored at -80 °C until further analysis by General Estradiol (E2) ELISA Kit (Bioss Inc, Woburn, MA, USA). The samples were anonymised before being analyzed. Prior to measurement, the samples were diluted to the appropriate range with PBS solution, and the concentrations of oestradiol were determined using a Thermo-Fisher Multiskan Go microplate reader (Thermo-Fisher, Waltham, MA, USA).

2.11. Statistical Analysis

For statistical analysis, GraphPad Prism 6 software was used. Dates are presented as means $\pm$ SD. Two-way ANOVA with Tukey post-test was used in phase-solubility study. In the case of the MTT test and macrophage activation test, one-way ANOVA and

Tukey post-test was used to compare the results. During the in vivo pharmacokinetic test, Kruskal–Wallis and Dunn post-test were used.

3. Results

3.1. Cytotoxicity Test

The cytotoxic effects of the PEG- β -CD polymer were evaluated using the MTT test on RAW 264.7 cell line and NIH/3T3 fibroblast cell line (Figure 1). The NIH-3T3 cell line was chosen because the cell line is a stable, reproducible, sensitive model for detecting cell-damaging effects and is also a good representation of the cell types that the parenteral preparation first encounters in vivo [26,29]. The effect on macrophages is important in the study of immune responses because they are the primary presenting cells. The RAW 264.7 cell line is a stable, robust, gold standard cell line suitable for phagocytosis, cytokine production, and antigen presentation studies [30]. The PEG- β -CD polymer was not cytotoxic on RAW 264.7 cells and NIH/3T3 fibroblast cells after 24 h of incubation up to 10.0 *w/w*% concentration the carrier did not cause significant damage on the main cells of the subcutaneous region, nor did it cause macrophage necrosis.

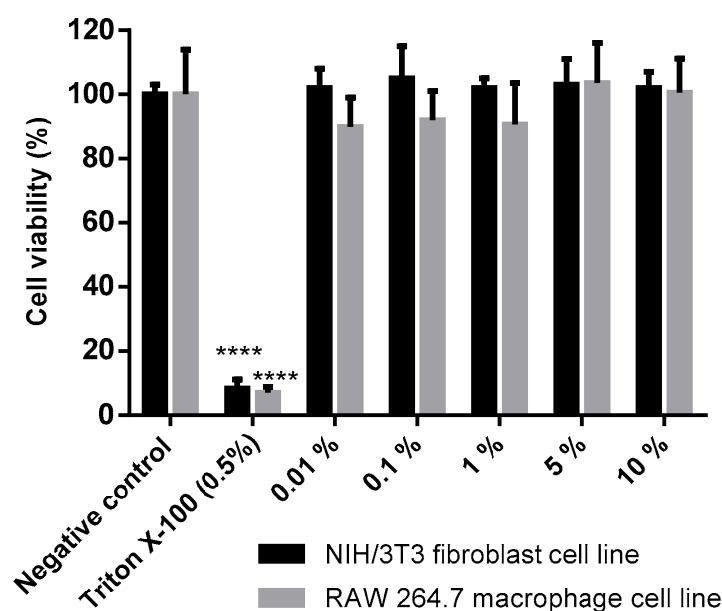


Figure 1. Cytotoxicity assay (MTT) confirmed that the PEG- β -CD polymer had no cell-damaging effect in the concentrations used (*w/w*%). Triton X-100 was used as a positive control and PBS solution as a negative control. (Data is presented as means $\pm$ SD; *n* = 3, **** indicates statistically significant differences at *p* < 0.0001).

3.2. Phase-Solubility Test

The phase-solubility test was performed to confirm the solubility-enhancing effects. Based on the phase-solubility curves (Figure 2), PEG cannot be used to increase the solubility of oestradiol in that concentration. When using β CD, an A_N -type curve is observed, the curve flattens, and the precipitation of the formed complex is observed to form an over-saturated solution after 0.3 m/m%. There is no significant improvement with the concomitant use of PEG and β -CD. In case of PEG- β -CD polymer, the line is A_L -type, and the solubility of oestradiol increased with increasing polymer concentration. Significantly better solubility was obtained, although the CD content of the polymer was only around 50%, confirming the important synergy of the high hydrodynamic volume of the cross-linked PEG- β -CD polymer. Thus, to focus on β CD component, it can be said to have increased solubility of oestradiol by more than 2-fold, as if ternary complexation had been used.

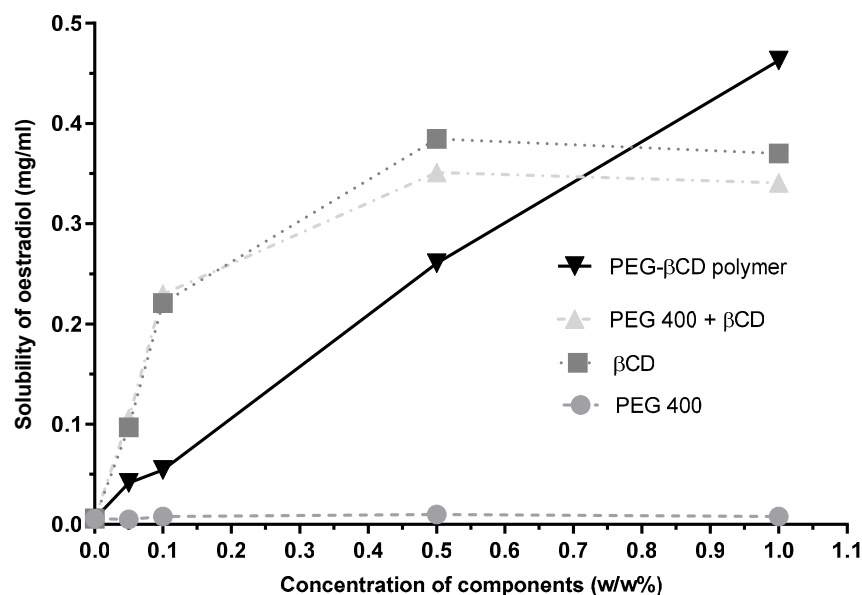


Figure 2. Phase-solubility curve presents the solubility of oestradiol (mg/mL) in water versus the m/m% concentration of the PEG-β-CD polymer (black triangle), βCD (square), PEG 400 (dot) and βCD and PEG 400 1:1 mixture (grey triangle). Statistically significant differences were observed between βCD and the PEG-β-CD polymer at concentrations of 0.05, 0.1, 0.5, and 1.0 w/w% ($p < 0.0001$). Similarly, PEG 400 + βCD differed significantly from the PEG-β-CD polymer at the same concentrations (0.05, 0.1, 0.5, and 1.0 w/w%; $p < 0.0001$). (Data is presented as means $\pm$ SD; $n = 3$).

3.3. Dynamic Light Scattering Measurements of Complex

The average particle size and particle size distribution of the 0.5 m/m% solution of polymer and the created complex were measured by DSL. The sizes of the complexes formed (270.70 ± 10.10 nm) after complexation are several times bigger than the polymer (49.76 ± 0.43 nm). During complex formation, oestradiol associates with the polymer chains, resulting in the formation of a coherent colloidal solution. A cyclodextrin-based polymer exhibited a polydispersity index (PDI) of 0.283, indicating a relatively narrow particle size distribution and good structural homogeneity. Complexation with estradiol led to an increase in the PDI to 0.583, reflecting a pronounced broadening of the particle size distribution. This effect is attributed to relaxation and expansion of the polymer's three-dimensional architecture, which creates additional free volume for estradiol incorporation and yields a larger final complex.

3.4. Fourier-Transform Infrared Spectroscopy (FTIR)

Significant variations in the shape and stretching vibrations of O-H bonds ($3600\text{--}3200\text{ cm}^{-1}$), aromatic CH=CH stretching ($1600\text{--}1500\text{ cm}^{-1}$), C-H stretching ($2950\text{--}2850\text{ cm}^{-1}$) and some fingerprint regions ($1500\text{--}900\text{ cm}^{-1}$) demonstrate the entrapping of the drug into the polymeric structure (Figure 3).

Specifically, the O-H stretching absorption becomes broader and shifts toward lower wavenumbers in the complex (20 cm^{-1}), indicating the formation or strengthening of hydrogen bonds between oestradiol and cyclodextrin. Minor shifts (2 cm^{-1}) and reduced intensity in the C-H stretching region suggest the inclusion of the hydrophobic steroid backbone of oestradiol into the cyclodextrin cavity. Additionally, noticeable changes in the fingerprint region, particularly in the C-O and C-C vibration bands, further support complex formation. As expected, no new peaks appeared in the spectrum, confirming the physical interaction of the two molecules and “dissolution” of the drug into the 3D structure of the PEG-β-CD polymer [20].

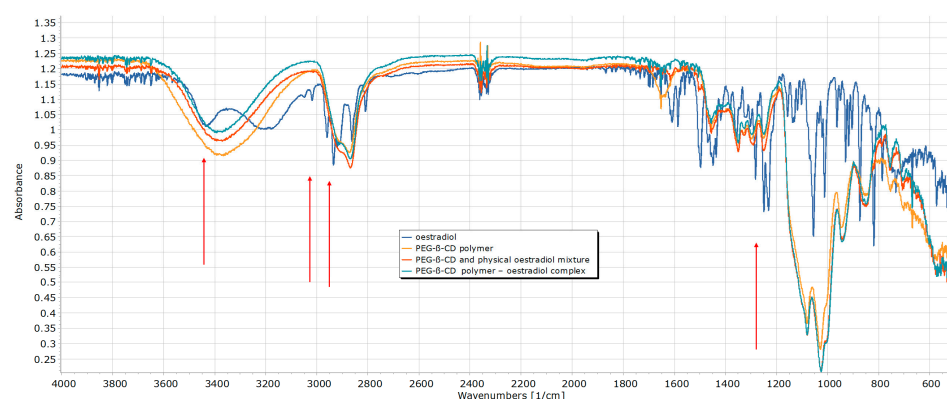


Figure 3. FTIR curve proved the complexation of oestradiol (chemical structure is in the lower left corner) by peak shifts. The PEG- β -CD polymer (yellow), oestradiol (blue), PEG- β -CD and physical oestradiol mixture (red) and PEG- β -CD polymer-oestradiol complex (green) are presented (data is presented as means; $n = 3$). The red arrow indicates the regions affected by the changes.

3.4.1. Macrophage Activation

In the macrophage activation test, MHC class II molecules are cell-surface glycoproteins that play a central role in the adaptive immune system by mediating antigen presentation to CD4⁺ T helper cells. The associated release of cytokines during this process triggers a broader immune response, which can result in rapid degradation and clearance of foreign substances. The PEG- β -CD polymer-oestradiol complex showed no significant effect on macrophage cells. Macrophages showed MHC-II receptor expression comparable to PBS-treated controls (negative control), whereas LPS treatment (positive control) significantly increased MHC-II expression (Figure 4).

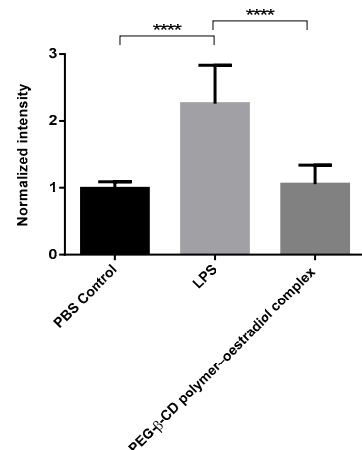


Figure 4. The response induced by LPS and the PEG- β -CD polymer-oestradiol complex is shown after normalization to the PBS control. PBS control is shown in black, LPS in grey, and the PEG- β -CD polymer-oestradiol complex in white. Data are presented as mean $\pm$ SD ($n = 3$). **** indicates a statistically significant difference at $p < 0.0001$.

The microscopic images (Figure 5) also confirm that macrophage cells were activated after LPS treatment. The uniform round blue cell nuclei indicate viable cells, which were stained by DAPI. The cell nuclei increasing in the case of LPS reflects increased cell activity, which can be explained by the chromatin structure opening wider compared with its original form. This chromatin state is associated with active mRNA transcription, which leads to the expression of genes encoding cell surface receptors, in this case the expression of MHC-II. The uniform round blue cell nuclei indicate viable cells, while the intensity of the green colour

indicates macrophage activation due to increased receptor presence. Fluorescent labelling of the LPS-treated cell surface was more intense compared to the control.

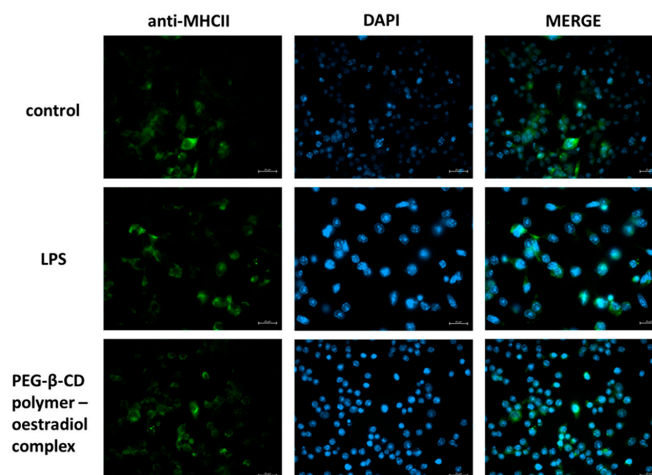


Figure 5. Fluorescence microscopic images of LPS-, PEG- β -CD polymer-oestradiol complex-treated and untreated macrophage cells (control). We stained nucleic acid-containing cell nuclei blue with DAPI, and anti-MHCII antibody was labelled with green. The uniform round blue cell nuclei indicate viable cells, while the intensity of the green colour indicates macrophage activation due to increased receptor presence. The scale bar indicates 20 μ m.

3.4.2. Inflammatory Factor Activation by RT-qPCR

Figure 6 displays the relative RNA expression of various cytokines (TNF- α , IL-6, IL-10, IL-1 α , IL-1 β) across four different treatment conditions: Untreated, LPS, IFN- γ , and Complex. The vertical axis represents the relative protein expression levels. For TNF- α , IL-6, and IL-10, protein expression levels are uniformly low across all treatment conditions. The Untreated, IFN- γ , and Complex conditions show very similar expression levels, indicating that these treatments do not significantly alter the expression of these cytokines. LPS treatment shows a slight increase in TNF- α and IL-6 expressions compared to the other treatments, but this increase is marginal. A significant increase in protein expression is observed in the LPS-treated group for both IL-1 α and IL-1 β . The other treatment groups (Untreated, IFN- γ , and Complex) show negligible expressions of IL-1 α and IL-1 β , like their expression levels for TNF- α , IL-6, and IL-10. PCR studies showed that macrophages do not produce inflammatory cytokines when the preparation is used.

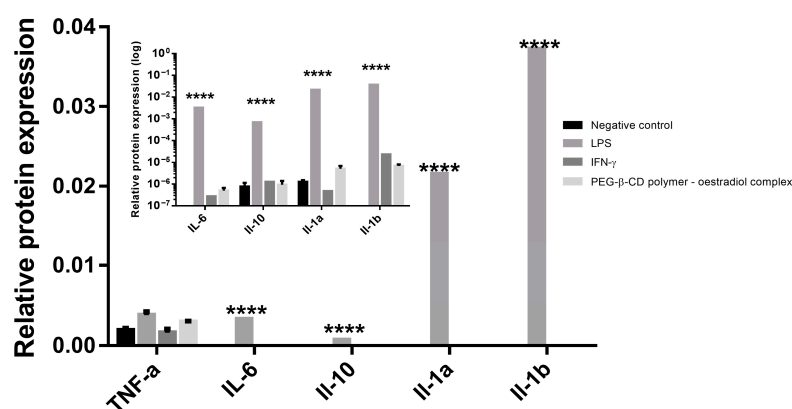


Figure 6. The changes in the cytokine amounts produced by macrophages after treatment with LPS, IFN- γ and PEG- β -CD polymer-oestradiol complex. PBS was used as negative control. The embedded figure shows the logarithm of the relative protein expression for comparability. (Data is presented as means; n = 3. **** indicates statistically significant differences at $p < 0.0001$).

3.5. *G. mellonella* Larvae Survivability Test

The safety of PEG- β -CD polymer-oestradiol complex was further evaluated using a larval model, which confirmed its non-toxic nature (Figure 7). Across all tested concentrations, the formulation did not induce significant mortality when compared to the control group. These findings suggest that the product is safe for use, even at higher concentrations. Additionally, the absence of any observable adverse effects in the larvae reinforces the potential for its application in broader biological contexts, supporting its safety profile for future research and practical use.

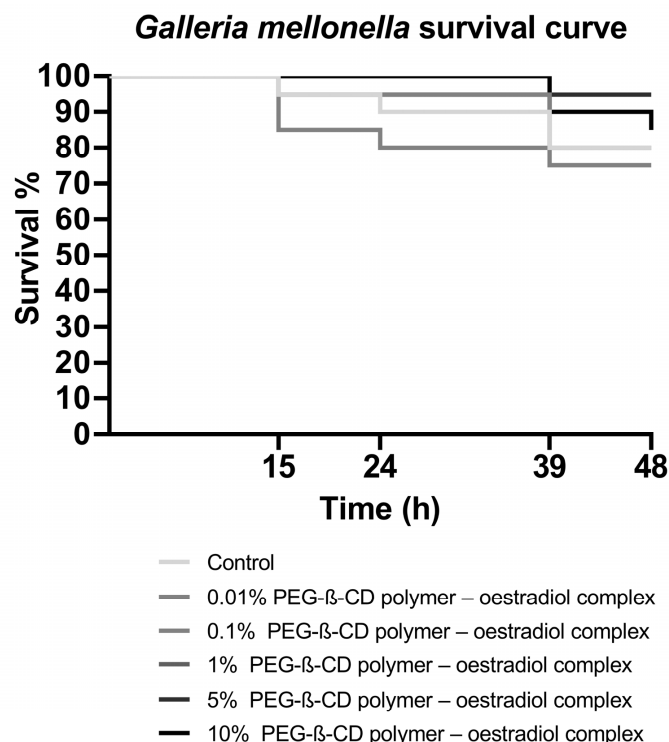


Figure 7. Larval survival within 48 h after administration of different percentage gels, demonstrating the safe use of the product. (n = 20).

3.6. *In Vivo* Pharmacokinetic Test

The active ingredient content of the formulation was determined by HPLC, as well as its osmolality and pH. The formulation was found to be near neutral pH, 7.03 pH. The solution was hypertonic, and the osmolality of the sample was 373 mOsmol/L. The drug content was found to be 5.0 mg/mL.

In the *in vivo* pharmacokinetic study, there were no animal exclusions. On Figure 8 distinct absorption profiles were observed based on the route of administration. Following the delivery of oestradiol, a very high peak concentration was reached within the first 24 h in the treatment groups. Specifically, intramuscular (IM) administration resulted in a substantial early spike, with serum levels reaching approximately 200–300 pg/mL on the first day. In contrast, subcutaneous (SC) administration produced a more moderate initial elevation, peaking at approximately 10–20 pg/mL. By the end of the first week, a sharp decline in serum concentration was observed in both treatment groups. Although the average plasma concentration remained slightly higher than that of the negative control rats during this first week, levels subsequently returned to baseline. From 7th day through 28th day, the oestradiol concentrations for both SC and IM routes remained low and stable (averaging 0.7–1 pg/mL and <1 pg/mL, respectively), effectively converging with the control group's baseline fluctuations.

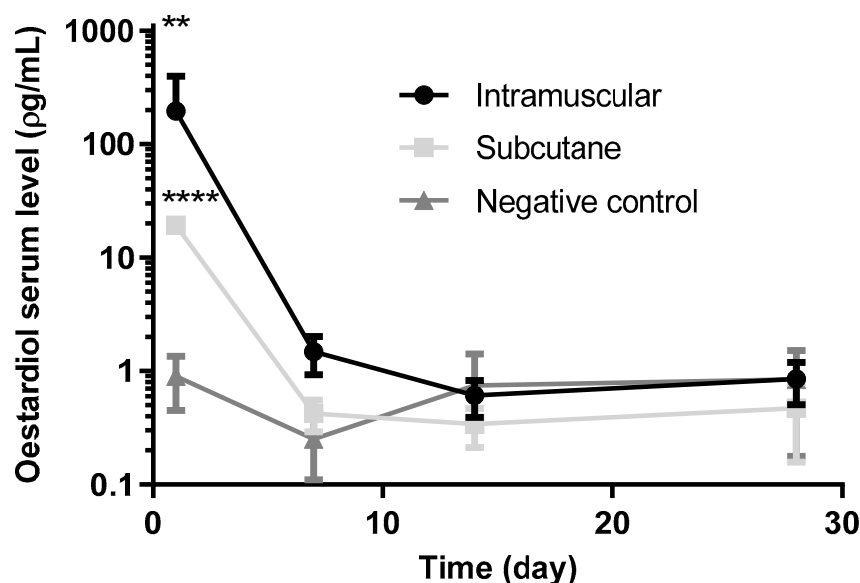


Figure 8. Changes in oestradiol plasma levels over the course of one month of treatment are presented. The results are organised in blocks of three showing the subcutaneous and intramuscular treatment and a control at 1, 7, 14 and 28 days (data is presented as means; $n = 6$, **** and ** indicate statistically significant differences at $p < 0.0001$ and $p < 0.01$).

4. Discussion

In this study, various experimental assays, including cytotoxicity, solubility, particle size distribution, and in vivo studies, feature the potential of PEG- β -CD polymer, a water-soluble polyethylene glycol- β -CD cross-linked polymer, in oestradiol formulations for menopause treatment. The complex can offer new solutions as a submicron carrier, unlike oestradiol salts or oils. Using it as a complex reduces side effects and can result in better patient compliance.

The cytotoxicity assays on RAW 264.7 macrophage cells and NIH/3T3 fibroblasts indicate that PEG- β -CD polymer presents minimal cytotoxicity up to 10% (w/w). The choice of NIH/3T3 fibroblasts as a model is particularly relevant, as these cells are representative of the subcutaneous tissue environment where the formulation is intended to act [31,32]. The RAW 264.7 cell line is a widely accepted immune cell model and a robust tool for analysing cytokine production, phagocytosis, and antigen presentation [30]. These results are in accordance with and support previous studies that have demonstrated the biocompatibility of monocyclodextrin-based carriers. For instance, studies by Loftson have shown that cyclodextrins generally possess low toxicity profiles [33], making them suitable for various biomedical applications such as compression, capsules or injections. In the case of cyclodextrin polymers and their modified derivatives, similar results were observed with this cell line; specifically, they exhibited no toxicity even at high concentrations [34–38], even though our previous results showed a cytotoxicity of high concentration for homogeneous monolayer cell types with our polymer [20]. The absence of significant cytotoxic effects on macrophages and fibroblast is crucial, as it suggests that the polymer does not trigger an adverse immune response, thereby ensuring its safety for therapeutic use.

The phase-solubility study reveals that the PEG- β -CD polymer significantly enhances the solubility of oestradiol compared with PEG, β -cyclodextrin (β -CD), and their physical mixtures. A dual inclusion mechanism is proposed for the PEG- β -CD polymer, involving (I.) entrapment of oestradiol within the β -CD cavity and (II.) diffusion of oestradiol into the free volume of the three-dimensional polymeric structure. The solubility enhancement observed for the PEG- β -CD polymer-oestradiol system follows an A_L -type phase-solubility

profile, indicating a linear increase in solubility with increasing polymer concentration. Previous studies have primarily investigated the effect of β -CD at lower concentrations, where a linear relationship between cyclodextrin concentration and oestradiol solubility was reported [39,40]. However, precipitation has been observed at the upper end of the β -CD solubility range, where increasing cyclodextrin concentrations lead to complex precipitation and result in an A_N -type phase-solubility profile [18]. The highest solubility enhancement has been reported for methyl- β -cyclodextrin (M- β -CD) [18], and in comparison, the solubility increase achieved in the present study is more modest. In the case of PEG 400, the previous literature indicates that the solubility of oestradiol increases with increasing solvent concentration [41], but only at higher PEG concentrations. At the concentration used in this study, the solubility enhancement was limited and not statistically significant. Meanwhile using the native β -CD and PEG cross-linker polymer agent together, we obtained poorer solubility, which has been previously described with other polymers [42]. Overall, the 1.5-fold increase in oestradiol solubility achieved with the PEG- β -CD polymer demonstrates its potential to improve drug bioavailability, particularly for poorly water-soluble compounds such as oestradiol.

The dynamic light scattering (DLS) analysis indicates a significant increase in particle size upon complexation of oestradiol with the polymer, with the average size of the complex reaching approximately 270.70 nm, compared to the polymer alone (49.76 nm) and confirming the importance of the high hydrodynamic volume offered by the 3D PEG- β -CD polymer structure. This size increase is indicative of successful complex formation, where multiple polymer chains may be linked by oestradiol molecules, forming a larger colloidal assembly [43]. Such a size range is typical for submicron particulate drug delivery systems, which are known to enhance drug stability and facilitate controlled release [44]. PDI also increases because of the heterogeneous complex formation, likely arising from non-uniform drug loading, polymer aggregation, or the coexistence of multiple populations (free polymer, partially loaded complexes, and fully loaded complexes). Such behaviour is commonly reported in supramolecular drug-carrier systems and reflects changes in intermolecular interactions and self-assembly upon guest molecule inclusion [43].

Fourier-transform infrared (FTIR) spectroscopy further confirms the proven the entrapment of the drug into the polymeric structure and complex formation, as evidenced by significant variations in the shape and stretching vibrations of O-H bonds, aromatic stretching and C-H stretching of oestradiol molecule. As expected, no new peaks appeared in the spectrum underlying the physical interaction of the two molecules with “dissolution” of the drug into the 3D structure of the PEG- β -CD polymer [45].

Macrophage activity studies have demonstrated that the oestradiol-cyclodextrin polymer complex does not significantly alter the expression of MHC-II receptors on macrophages, which are key markers of immune activation. This observation is particularly important, as it indicates that the formulation does not trigger a classical immune response, which could otherwise result in detrimental inflammatory reactions. In general, cyclodextrins have an immunomodulatory effect [25,46], although in the case of vaccines, in addition to the carrier, an adjuvant effect has also been described in the case of HP- β -CD, through macrophage activation [47–50]. Notably, cyclodextrin derivatives and cyclodextrin-based nanoparticles have been reported to exhibit intrinsic immunomodulatory and anti-inflammatory properties, including the modulation of macrophage phenotypes and the suppression of pro-inflammatory markers such as TNF- α , IL-1 β , and IL-6 in macrophage models, relative to soluble β -cyclodextrin controls [51,52]. In subcutaneous administration, minimizing local inflammation is essential to ensure tolerability and to prevent harmful tissue reactions. The results of this study support the safe use of the formulation in this context. In particular, the absence of significant changes in the expression and secretion

of pro-inflammatory cytokines, as demonstrated by RT-qPCR and protein assays, further confirms the immunocompatibility of the oestradiol–cyclodextrin polymer complex. Moreover, the minimal immunogenicity and low inflammatory profile of cyclodextrin polymers with various cross-linking agents have been previously reported in the literature, underscoring their suitability for drug delivery applications where immunological neutrality is critical [53].

The *Galleria mellonella* larval model used to assess the safety of the formulation confirms its non-toxic nature, with no significant mortality observed across tested concentrations. This model is increasingly recognised for its utility in preclinical safety testing, offering a cost-effective and ethically favourable alternative to traditional mammalian models [54]. The absence of adverse effects in the larvae supports the formulation's safety profile and suggests its potential applicability in broader biological contexts. Furthermore, the physical parameters of the product confirmed its safety and pharmacopoeia suitability for animal studies.

The oestradiol concentration–time profiles observed in our study are consistent with previously published pharmacokinetic data on injectable oestradiol esters. In both our data and the literature, oestradiol levels exhibit a pronounced early peak shortly after administration, followed by a rapid decline over the first 7–10 days and a subsequent lower, more stable phase toward the end of the dosing interval. Maximum plasma level peak was observed in the first day after administration in both cases, and the effect wears off after the first week. The intramuscular route yields more encouraging results due to a more predictable and clinically relevant exposure profile of the active ingredient as subcutaneous, and because its administration is similar to commercially available formulations. This characteristic pattern is consistent with the well-established release and hydrolysis kinetics of long-acting oestradiol esters [9,55,56].

Comparison of the different study's curves indicates strong qualitative agreement in the overall shape and temporal dynamics of the oestradiol profiles. Although absolute oestradiol concentrations differ between studies, this is likely due to variations in dose, ester formulation, administration route, interindividual pharmacokinetic variability, the magnitude and timing of peak levels, and the subsequent rate of decline observed in our results. When oestradiol esters are administered intramuscularly, their duration of action differs according to the ester moiety. Oestradiol benzoate produces a relatively short-lasting effect, with peak serum concentrations occurring approximately 24 h after injection and declining by days 4–5 [57–62]. Oestradiol valerate exhibits a longer duration of action, reaching peak serum levels around day 2 and gradually declining over approximately two weeks [63–67]. Among these esters, oestradiol cypionate has the longest duration of action, with peak serum concentrations typically observed 5–7 days after administration and serum levels decreasing over a period of up to one month [9,68–70]. These findings support the utility of the developed formulation. The oestradiol levels achieved are comparable to those obtained with oestradiol esters, suggesting that it may represent a viable alternative to oestradiol benzoate and oestradiol valerate.

In summary, an oestradiol–cyclodextrin polymer complex was successfully developed as a potential alternative to conventional oestradiol oil ester formulations, with the goal of improving tolerability and reducing side effects. The complex enhances oestradiol solubility, forms stable submicron assemblies, and exhibits minimal cytotoxicity and immunogenicity in vitro, with confirmed safety in the *Galleria mellonella* model. Particle size and spectroscopic analyses support successful complex formation via a dual inclusion mechanism. Pharmacokinetic profiles following intramuscular administration are comparable to conventional oestradiol esters, suggesting this formulation may provide a safer, better-tolerated alternative to existing oil-based therapies.

5. Conclusions

A stable oestradiol complex based on a PEG- β -cyclodextrin polymer was developed that demonstrates minimal cytotoxicity and excellent immunocompatibility in vitro and in vivo. The complex enhances the solubility of oestradiol and forms submicron particles of suitable size for controlled drug delivery. Pharmacokinetic profiles are comparable to those of established oestradiol esters while potentially reducing inflammation-related side effects. Overall, this formulation represents a promising alternative to conventional oestradiol benzoate and valerate preparations, offering improved safety and patient compliance. The study expands the current knowledge of cyclodextrin polymers and may inform future research toward their potential pharmaceutical application.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

CD	Cyclodextrin
PEG- β -CD polymer	Cross-linked β -cyclodextrin with polyethylene glycol diglycidyl ether
β CD	Beta-cyclodextrin
PEG 400	Polyethylene glycol 400
MTT assay	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay
NIH-3T3	Mouse fibroblast cell line
RAW 264.7	Mouse macrophage cell line
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal bovine serum
HPLC	High-performance liquid chromatography
DAD	Photodiode array detector
DLS	Dynamic light scattering measurements
FTIR	Fourier-transform infrared spectroscopy
ATR	Attenuated Total Reflectance
LPS	Lipopolysaccharide
PBS	Phosphate-buffered saline solution
DAPI	4',6-diamidino-2-phenylindole

RT-qPCR	Reverse transcription quantitative polymerase chain reaction
IFN- γ	interferon-gamma
IL	interleukin
TNF- α	Tumour necrosis factor alpha
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
PDI	Polydispersity index
SC	subcutaneous
IM	intramuscular

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