



Research Article

Preparation and Characterization of Methotrexate Loaded Polymeric Nanoparticles by Nanoprecipitation Technique

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Abstract

The aim of this study was to develop and characterize methotrexate-loaded polymeric nanoparticles by nanoprecipitation technique. Eudragit® S 100 and ethyl cellulose were employed to develop methotrexate-loaded nanoparticles by nanoprecipitation technique. Six different formulations (f1, f2, f3, f4, f5 and f6) were prepared with each polymer by varying the drug to polymer ratios (1:1, 1:1.5, 1:2, 1:3, 1.5:1 and 2:1). Dimethyl sulfoxide (DMSO) was used as a solvent and Tween® 20 as a surfactant. Among the six formulations of polymeric nanoparticles prepared by nanoprecipitation technique, F4 formulation was considered as a best formulation with each polymer. Based on comparison results of mean particle diameter, zeta potential, drug content and entrapment efficiency, Eudragit® S 100 was considered to be the most suitable polymer for preparation of methotrexate-loaded nanoparticles by nanoprecipitation technique. Based upon the evaluation studies, the best formulation was characterized for scanning electron microscopy (SEM), particle size, zeta potential and anti-cancer activity in MCF-7 cell line by MTT assay.

Keywords: Methotrexate; Polymeric nanoparticles; Nanoprecipitation technique

Introduction

A conventional preparation like solutions, suspensions or emulsions for drug delivery has certain restrictions like high dose and low bioavailability, intolerance, instability. They show some changes in the blood plasma drug levels and do not provide sustained effect. Due to the attendance of acidic and basic medium in the body, it is essential that every drug should reach the target site without any alteration in its physical and chemical properties [1, 2]. The major focus on Novel drug delivery systems during the past two decades is to improve the therapeutic

efficacy and safety profile of the drug substances. Among all the colloidal systems, nanoparticles hold promise as drug delivery through various routes due to their greater stability and easier manufacturing ability. And also since many years, there has been a shift in drug delivery research from micro to nanoscale. Thus, nanotechnology is evolving as a promising field in medicine that shows significant therapeutic benefits. These systems are used for specific drug delivery, controlled drug delivery and also for the improvement of bioavailability of the hydrophobic drugs [3, 4].

Methotrexate (MTX), a dihydrofolate reductase inhibitor, is one of the most widely used drugs for the

treatment of various neoplastic diseases. It is used in the treatment of certain cancers like breast cancer, skin and lung cancer. Clinical studies have revealed that the curative effect of MTX tablet regarding cancers was limited due to its toxic dose-related side effects to normal cells, nephrotoxicity, immunosuppressant, bone marrow suppression, and ulcerative stomatitis, acute and chronic hepatotoxicity and also due to the drug resistance of the tumor cells. Hence, there is a need to develop methotrexate nanoparticles in order to minimize the adverse effects associated with the MTX tablet dosage form [5, 6].

Nanoprecipitation is also called solvent displacement or interfacial deposition. It is considered as one of the first developed techniques used for the encapsulation of drug molecules [7]. This technique was developed by Fessi et al. (1989). Although, several techniques have been used for the preparation of submicron particles from preformed polymers, nanoprecipitation is regarded as a quite simple and reproducible technique that allows the obtaining of submicron-sized polymer particles [8, 9].

In the present study, attempts have been made to prepare methotrexate nanoparticles by using Eudragit® S 100 and ethyl cellulose (EC) as a polymer as these are low cost polymer and can formulate the methotrexate nanoparticles in academic research.

Experimental

Materials

Drug: Methotrexate, gift sample from Mac-Chem Products (India) Pvt. Ltd., Mumbai.

Polymers: Eudragit® S 100, ethyl cellulose obtained from SD Fine Chem. Limited, Mumbai.

Solvent: DMSO, acetone obtained from SD Fine Chem. Limited, Mumbai.

Stabilizer: Tween® 20 obtained from SD Fine Chem. Limited, Mumbai.

Methods

Methotrexate loaded polymeric nanoparticles were prepared by nanoprecipitation technique. Eudragit® S 100 and ethyl cellulose was selected as a polymer for this technique. DMSO was used as a solvent [10].

Nanoprecipitation

Six different formulations (F1, F2, F3, F4, F5, and

F6) were prepared by varying the drug to polymer ratio. Weighed quantities of Methotrexate and Eudragit® S100 / ethyl cellulose were dissolved in 10 mL of dimethyl sulphoxide. (Ethyl cellulose was dissolved in acetone before adding DMSO). The organic phase was then added to 25 mL of aqueous phase containing 0.1% Tween® 20 surfactant with continuous stirring at 500 rpm. The appearance of precipitate was considered as the end point. After the attainment of endpoint, the solution was kept for stirring for 5 hours. The formed precipitate was separated from the solution by means of vacuum rotary evaporator. The obtained precipitate was air-dried to completely remove the moisture content [11, 12].

Table 1 Formulation ratios of methotrexate loaded ES 100 / ethyl cellulose nanoparticles

Formulation	Ratio of drug to polymer used	Optimized parameters
F1	1:1	
F2	1:1.5	DMSO
F3	1:2	0.1% v/v Tween® 20
F4	1:3	500 rpm
F5	1.5:1	5 h
F6	2:1	

Evaluation studies [13]

The obtained formulations of technique are evaluated for the following parameters.

Determination of drug content

Free drug of the formulations was first determined in the supernatant by choosing a solvent in which only the free drug got dissolved and not the other ingredients. To determine the drug content, 50 mg drug equivalent to formulation was weighed accurately and transferred into 100 mL beaker containing 50 mL of DMSO. The solution was stirred at 700 rpm for 3 h by using magnetic stirrer. The resultant solution was filtered and the amount of the drug in the filtrate was estimated after suitable dilution by UV spectrophotometer at 303 nm [14, 15].

Entrapment efficiency [16]

Entrapment efficiency indicates the amount of drug encapsulated in the formulation. The method of choice for entrapment efficiency determination is separation of free drug by ultracentrifugation, followed by quantitative analysis of the drug from the formulation. The samples were centrifuged by using ultracentrifuge at 17000 rpm for 40 min.

Percentage entrapment efficiency may be calculated from the following formula:

Entrapment efficiency = (Amount of drug encapsulated in the formulation / Total amount of drug in the formulation) \times 100.

Loading capacity [17]

It indicates the capacity of the polymer to load a drug.

Loading capacity = [(Total amount of drug – Amount of free drug concentration) / Nanoparticles weight] \times 100.

In-vitro drug release kinetics [18, 19]

In-vitro drug release studies were conducted by means of orbital shaker. 50 mg of each accurately weighed formulation was transferred into 250 mL conical flask containing 50 mL pH 7.4 phosphate buffer. They were kept in an orbital shaker at 100 rpm maintained at 37 °C. Aliquots of 2 mL buffer were withdrawn at predefined time intervals and the medium was replaced with same volume of buffer. This study was carried out for 12 h, and the amount of drug release was estimated by determining the absorbance at 303 nm using Elico UV spectrophotometer.

Characterization of nanoparticles [20]

Particle size analysis and zeta potential measurement [21, 22]

The average particle size and size distribution of MTX polymeric nanoparticles were determined by dynamic light scattering (DLS), using Malvern Zetasizer. The zeta potential (surface charge) which indicates the stability of the nanoparticle's can be defined as electrokinetic potential that is determined by electrophoretic mobility. Samples were prepared by diluting with water and corresponding zeta potential was measured using Malvern Zeta Sizer.

Determining the size and morphology of the nanoparticles [23, 24]

Scanning electron microscopy (SEM) was used to determine the shape, size and surface morphology of the MTX polymeric nanoparticles. Nanoparticulate suspension is made to obtain photomicrographs using SEM.

In vitro cytotoxicity assay

Aim

To determine the Anti-cancer activity of test

compounds in vitro by MTT Assay.

Materials and methods

Dulbecco's modified Eagles medium (DMEM), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], trypsin, and EDTA phosphate buffered saline (PBS) were purchased from Sigma Chemicals Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Gibco, 25 cm² and 75 cm² flask and 96 well plated purchased from Eppendorf India [25, 26].

Procedure

Cell viability was evaluated by the MTT Assay with three independent experiments with six concentrations of compounds in triplicates. Cells were trypsinized and perform the trypan blue assay to know viable cells in cell suspension. Cells were counted by haemocytometer and seeded at density of 5.0×10^3 cells / well in 100 μ L media in 96 well plate culture medium and incubated overnight at 37 °C. After incubation, take off the old media and add fresh media 100 μ L with different concentrations of test compound in the wells in 96 plates. After 48 h., Discard the drug solution and add the fresh medic with MTT solution (0.5 mg/mL) was added to each well and plates were incubated at 37 °C for 3 h. At the end of incubation time, precipitates are formed as a result of the reduction of the MTT salt to chromophore formazan crystals by the cells with metabolically active mitochondria. The optical density of solubilized crystals in DMSO was measured at 570 nm on a microplate reader. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% values is generated from the dose-response curves for each cells using with origin software [27, 28].

Inhibiiton (%) = [(Control – Treatment) / Control] \times 100

Results and Discussion

The obtained formulations were evaluated for the above mentioned parameters and the results are discussed as follows.

Evaluation results of methotrexate-Eudragit® S 100 nanoparticles

Drug content

All the prepared formulations of MTX loaded

Eudragit® S100 nanoparticles were evaluated for drug content.

Drug content of the formulations, F1-F6 was found to be $72\pm 2\%$, $72.6\pm 3\%$, $80.4\pm 2\%$, $93.1\pm 3\%$, $77.2\pm 2\%$, and $62.6\pm 2\%$, respectively. These results revealed that all the formulations showed good drug content and F4 formulation showed the highest percentage of drug content, i.e.; $93.1\pm 3\%$.

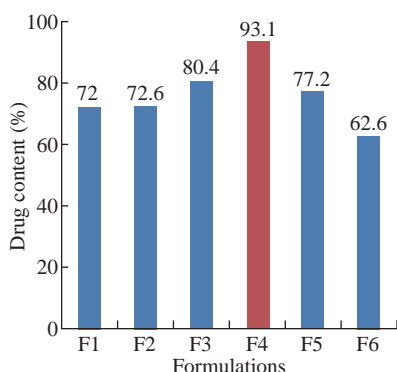


Fig. 1 Drug content of methotrexate-Eudragit® S100 nanoparticles.

Entrapment efficiency

The entrapment efficiency of all the formulations, i.e. F1-F6, was determined to be $77.09\pm 2\%$, $79.19\pm 3\%$, $80.06\pm 2\%$, $82.5\pm 3\%$, $72.75\pm 1\%$ and $74.9\pm 2\%$, respectively. As the amount of polymer was increased from F1 to F4 formulation, the encapsulation efficiency was increased. However, on increasing the drug concentration, there was decrease in entrapment efficiency that could be due to an inadequate amount of polymer present in the system being insufficient to entrap the drug inside the matrix.

Thus, the optimum drug encapsulation was found to be for F4 formulation showing i.e.; 82.5% .

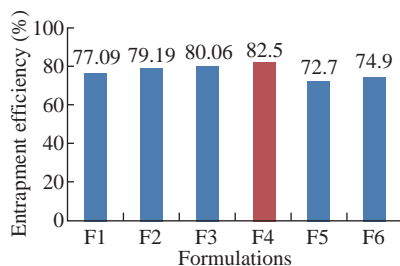


Fig. 2 Drug entrapment efficiency of methotrexate-Eudragit® S100 nanoparticles.

Loading capacity

Loading capacity of all the formulations, F1-F6 was

found to be 26.9% , $27.4\pm 1\%$, $25.04\pm 2\%$, $16.6\pm 1\%$, $33.3\pm 2\%$ and $29.58\pm 2\%$, respectively. By comparison, F5 formulation showed higher loading capacity than other formulations.

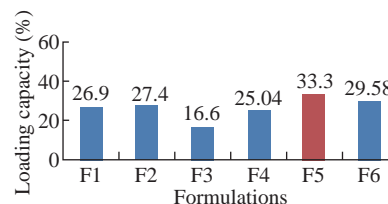


Fig. 3 Loading capacity of Methotrexate-Eudragit® S100 nanoparticles.

In-vitro drug release studies

The in-vitro drug release studies were performed for all the formulations of MTX loaded ES 100 nanoparticles in 7.4 pH phosphate buffer conducted by means of orbital shaker (Orchid, Scientifics) for a time period of 12 h.

Among all the formulations, the drug release from F4 formulation was good and the release was sustained up to 12 h, i.e.; $89.8\pm 3\%$ in 12 h.

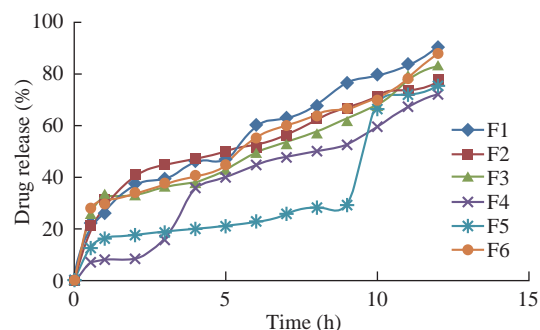


Fig. 4 In-vitro drug release profile of methotrexate-Eudragit® S100 nanoparticles.

Evaluation results of methotrexate-ethyl cellulose nanoparticles

Drug content

Drug content of the formulations F1-F6 was found to be $68.9\pm 2\%$, $69.02\pm 2\%$, $70.6\pm 3\%$, $75\pm 3\%$, $70.2\pm 2\%$ and $82.3\pm 2\%$, respectively. These results revealed that all the formulations showed good drug content and F6 formulation showed the highest percentage of drug content, i.e.; $82.3\pm 2\%$.

Entrapment efficiency

Entrapment efficiency of all the formulations, F1-F6 was determined to be $61.4\pm 2\%$, $65.9\pm 2\%$, $74.2\pm 3\%$, $79.3\pm 2\%$, $59.5\pm 2\%$ and $53.4\pm 3\%$, respectively.

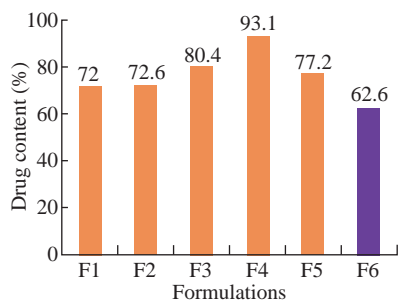


Fig. 5 Drug content of methotrexate-ethyl cellulose nanoparticles.

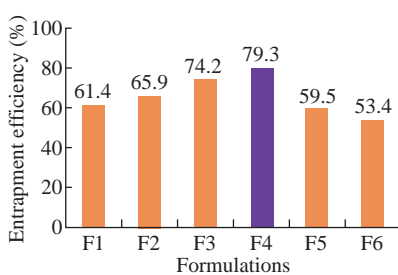


Fig. 6 Drug entrapment efficiency of methotrexate-ethyl cellulose nanoparticles.

Loading capacity

Loading capacity of all the formulations, F1-F6 was found to be $21.54 \pm 2\%$, $20.57 \pm 2\%$, $19.25 \pm 3\%$, $18.1 \pm 2\%$, $24.6 \pm 2\%$ and $29.7 \pm 2\%$, respectively. By comparison, F6 formulation showed higher loading capacity than the other formulations.

In-vitro drug release studies

Among all formulations F4 (1:3) formulation showed maximum drug release of $90.05 \pm 2\%$ in a time period of 12 h as shown in Fig. 8.

Average particle size

Size distributions of the prepared nanoparticles along the mean diameter was measured using particle size analyser. Average particle size of the prepared drug loaded Eudragit® S100 nanoparticles was recorded. The minimum value was found for F4 formulation as of 704.3 nm (Fig. 9).

Zeta potential

Zeta potential of the prepared drug loaded Eudragit® S100 nanoparticles was measured using zeta sizer. Nanoparticles of F4 formulation showed higher stability, bearing a value of -21.3 mV.

Average particle size and zeta potential

The size distributions of the prepared nanoparticles along the mean diameter were measured using particle

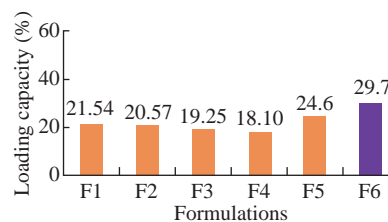


Fig. 7 Loading capacity of methotrexate-ethyl cellulose nanoparticles.

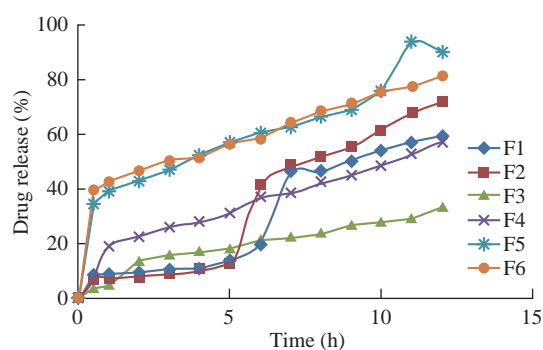


Fig. 8 In-vitro drug release profile of methotrexate-ethyl cellulose nanoparticles.

size analyser. The average particle size of the prepared drug loaded ethyl cellulose nanoparticles was recorded. The minimum value was found for F4 formulation as of 622.6 nm (Fig. 11 and 12).

Zeta potential

Zeta potential of the prepared drug loaded ethyl cellulose nanoparticles was measured using zeta sizer. Nanoparticles of F4 formulation showed higher stability, bearing a value of -23.3 mV.

In-vitro cytotoxicity assay

Based on the characterization and evaluation studies of the best formulations prepared by nanoprecipitation technique, MTX-EC (1:3) was considered as a best formulation and determine for the anticancer activity on MCF-7 cell line by MTT assay.

Cisplatin was taken as a standard and its IC_{50} value was observed to be $4.7 \mu\text{M}$.

The IC_{50} value for the given formulation (MTX-EC 1:3) was found to be $74.65 \mu\text{g}$.

Discussion

In the present study, attempts have been made to prepare methotrexate nanoparticles by using Eudragit® S100 and ethyl cellulose as a polymer as these are low cost polymer and can formulate the methotrexate nanoparticles in academic research. Methotrexate acts

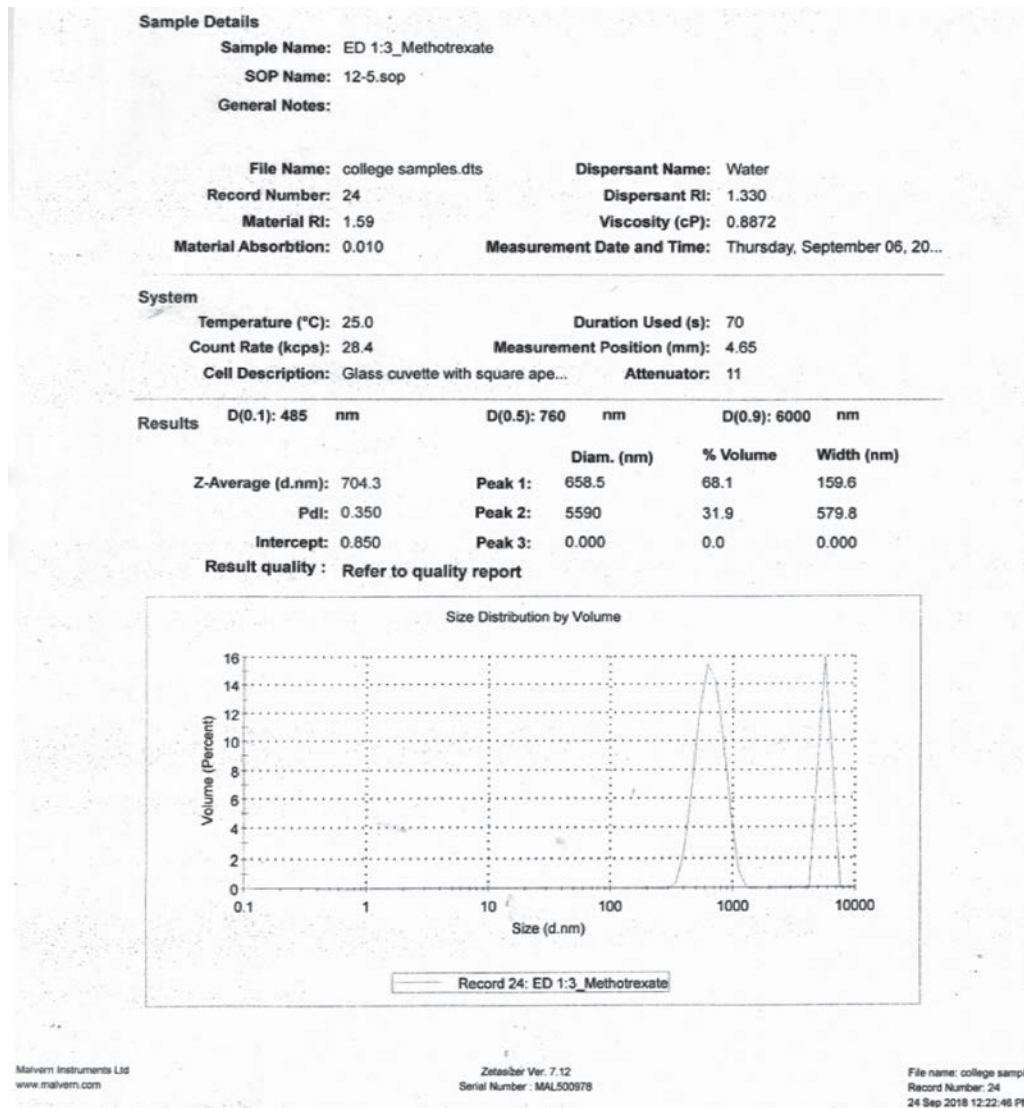


Fig. 9 Particle size analysis of F4 formulation.

Table 2 Cytotoxicity effect of F4 formulation (methotrexate-ethyl cellulose 1:3) on the growth of MCF-7 cell line

Concentration (μg)	OD at 570	Inhibition (%)	Viability (%)
5	0.281	20.62	79.38
10	0.175	50.56	49.44
25	0.177	50	50
50	0.167	52.82	47.18
75	0.163	53.95	46.05
100	0.192	45.76	54.24

as anti-cancer drug through its anti-metabolic effect by interfering with DNA and RNA growth by substituting for the normal building blocks of RNA and DNA. MTX is one of the most commonly used chemotherapeutic agent for human malignancies. Six formulations (1:1,

Table 3 IC_{50} value of methotrexate-EC 1:3 formulation

S. No.	Sample name	IC_{50} (μg) MCF7
1	ED (1:3)	74.65

1:1.5, 1:2, 1:3, 1.5:1 and 2:1) were prepared with each polymer by varying the concentrations of drug and polymer.

Out of six formulations of Eudragit® S100, F4 formulation was found to be the best formulation with drug content of 93.1%, entrapment efficiency of 82.5%, loading capacity of 25.04%, mean particle diameter of 704.3 nm, zeta potential value of -21.3 mV. In-vitro drug release data showed 89.8% of drug release controlled up to 12 h.

Out of six formulations of ethyl cellulose, F4 formulation was found to be the best formulation with

Sample Details

Sample Name: ED 1:3_Methotrexate 1
SOP Name: Zeta Potential DIP CELL.sop
General Notes:
File Name: college samples.dts
Record Number: 27
Dispersant Name: Water **Dispersant Dielectric Constant:** 78.5
Dispersant RI: 1.330 **Viscosity (cP):** 0.8872
Date and Time: Thursday, September 06, 2018 5:15:42 PM

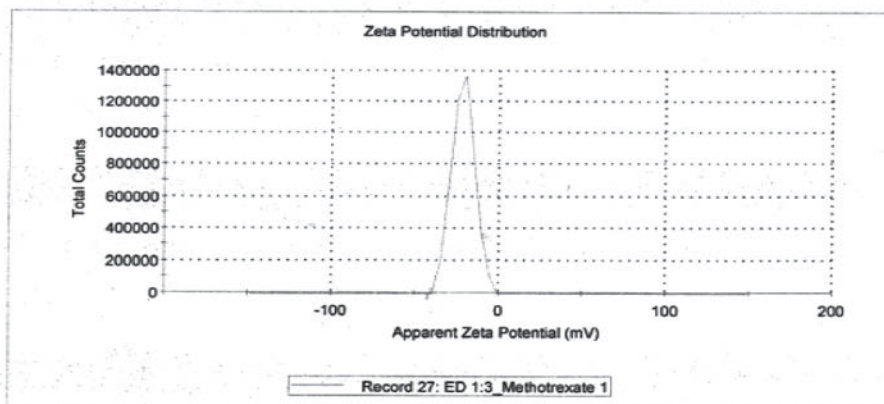
System

Temperature (°C): 25.0 **Zeta Runs:** 30
Count Rate (kcps): 297.7 **Measurement Position (mm):** 4.50
Cell Description: Zeta dip cell **Attenuator:** 7

Results

	Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -21.3	Peak 1: -21.3	100.0	6.63
Zeta Deviation (mV): 6.63	Peak 2: 0.00	0.0	0.00
Conductivity (mS/cm): 0.0352	Peak 3: 0.00	0.0	0.00

Result quality: Good



Malvern Instruments Ltd
www.malvern.com

Zetasizer Ver. 7.12
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Fig. 10 Zeta potential analysis of F4 formulation.

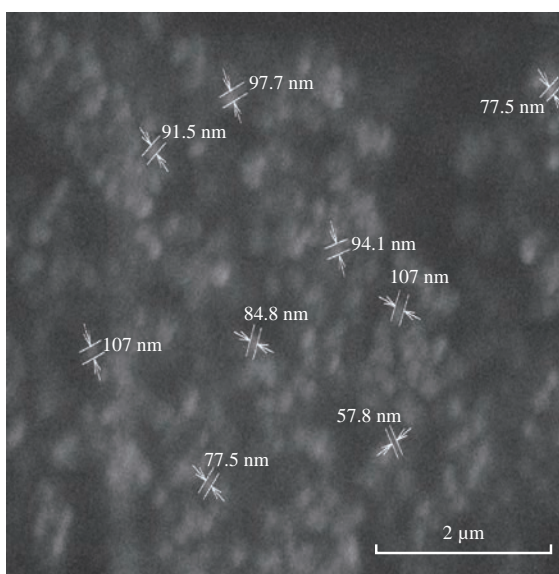


Fig. 11 SEM of F4 formulation.

drug content of 75%, entrapment efficiency of 79.3%, loading capacity of 18.1%, mean particle diameter of 622.6 nm, zeta potential value of -23.3 mV. In-vitro drug release data showed 90% of drug release controlled up to 12 h. In-vitro drug release studies and its data analysis have proven that all the formulations showed a sustained release that followed zero order and fickian type of diffusion pattern.

Based on the characterization and evaluation parameters, F4 (1:3) formulation using Eudragit® S100 as a polymer was found to be the best formulation by nanoprecipitation technique. This formulation was considered for determining anticancer activity by MTT assay in MCF-7 breast cancer cell line. From the results it was found that the formulation exhibited anticancer activity at an IC₅₀ value of 74.65 μg.

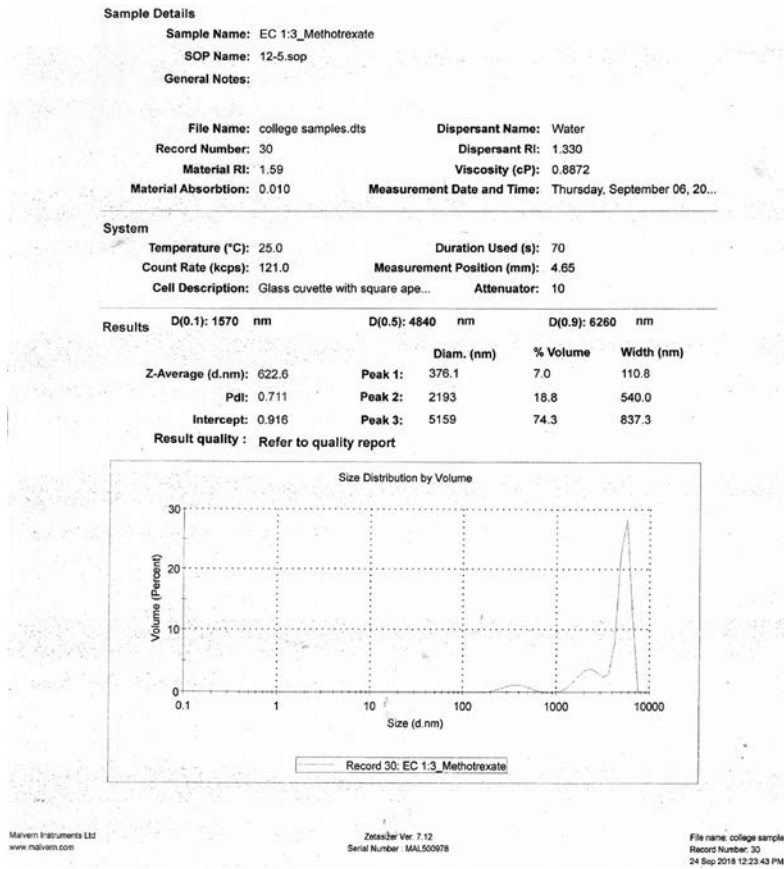


Fig. 12 Particle size analysis of F4 formulation.

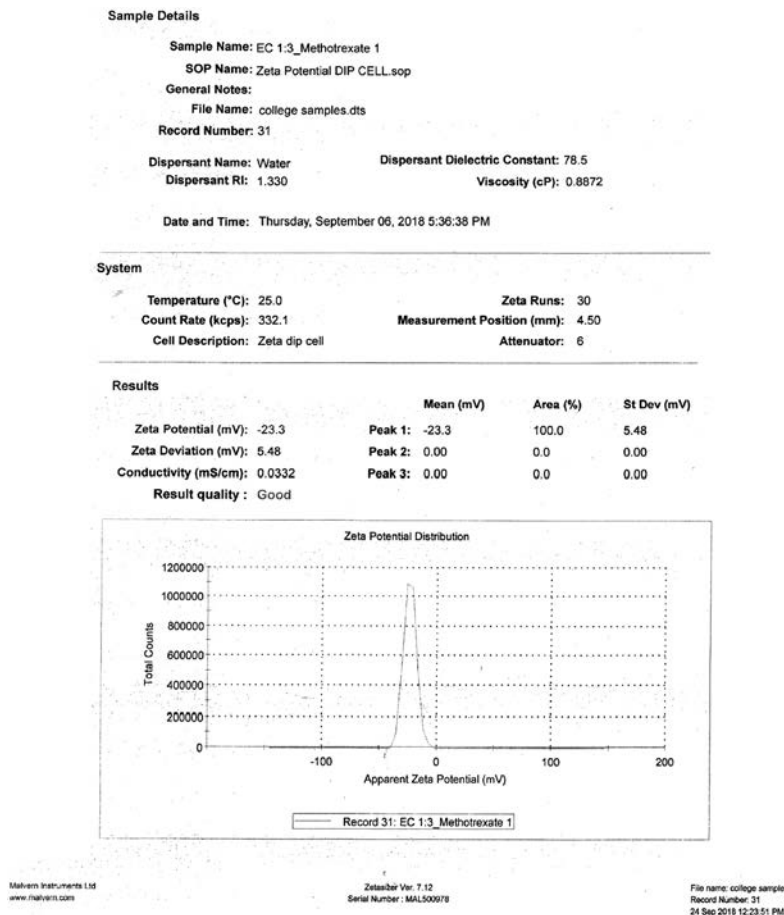


Fig. 13 Zeta potential analysis of F4 formulation.

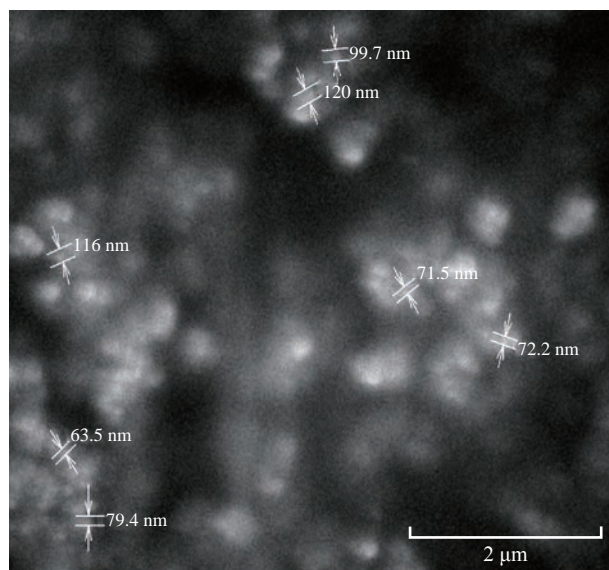


Fig. 14 SEM of methotrexate-ethyl cellulose F4 formulation.

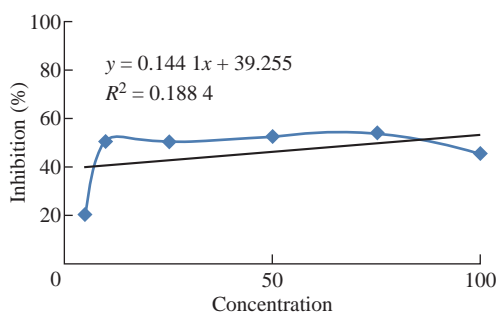


Fig. 15 Cytotoxicity effect of methotrexate-EC (1:3) on the growth of MCF-7 cell line.

Conclusions

Both polymers were compared for the characterization and evaluation parameters. By comparison, Eudragit® S100 was found to be a better polymer over ethyl cellulose for the preparation of methotrexate nanoparticles because of its smaller mean particle diameter (704.3 nm), higher stability (−21.3), greater entrapment efficiency (82.5%), and anti-cancer activity at an IC_{50} value of 74.65 μ g.

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