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Original article

# *In vitro* control release, cytotoxicity assessment and cellular uptake of methotrexate loaded liquid-crystalline folate nanocarrier

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ABSTRACT

Folate molecules self-assemble in the form of stacks to form liquid-crystalline solutions. Nanocarriers from self-assembled folates are composed of highly ordered structures, which offer high encapsulation of drug (95–98%), controlled drug release rates, active cellular uptake and biocompatibility. Recently, we have shown that the release rates of methotrexate can be controlled by varying the size of nanoparticles, cross-linking cation and cross-linking concentration. The present study reports the *in vitro* cytotoxic behavior of methotrexate loaded liquid-crystalline folate nanoparticles on cultured HeLa cells. Changing drug release rates can influence cytotoxicity of cancer cells. Therefore, to study the correlation of release rate and cytotoxic behavior, the effect of release controlling parameters on HeLa cells was studied through MTT assay. It is reported that by controlling the methotrexate release, the survival rates of HeLa cells can be controlled. Released methotrexate kills HeLa cells as effectively as free methotrexate solution. The co-culture based *in vitro* cellular uptake study through fluorescence microscopy on folate receptor positive and negative cancer cells shows that the present nanocarrier has the potential to distinguish cancer cells from normal cells. Overall, the present study reports the *in vitro* performance of self-assembled liquid-crystalline folate nanoparticles, which will be a platform for further *in vivo* studies and clinical trials.



## 1. Introduction

In the field of cancer research, in particular, nanoparticles are being studied as therapeutic and diagnostic tools to better understand, detect, and treat cancer. Nanoparticle mediated controlled chemotherapy is also one strategy, which claims to make conventional chemotherapy more effective. In general, conventional chemotherapy is effective for treatment of cancer, but due to direct administration of drug, patient experiences extremely high levels of drug in the body. These levels (higher than therapeutic levels required) are high enough to kill maximum number of cancer cells, but at the same time, these levels are also toxic to the normal healthy tissues of the body. Past studies have reported several serious off-target side effects [1,2]. At low doses, conventional chemotherapy will be ineffective against the tumor, whereas, at excessive doses, the toxicity will be intolerable to the patient. Moreover, there is a significant increment in these side effects with repetitive cycles of drug administration (alternative days, weeks etc.) [3,4].

Modified release pattern of chemotherapeutic drugs can enhance the therapeutic effects on malignant cells with reduced toxicity on normal cells by maintaining a constant therapeutic window. This can possibly be achieved through controlled drug delivery strategies. Controlled drug delivery is desirable in order to achieve multiple but constant therapeutic levels in chemotherapy for prolonged time without drug re-administration. This can reduce toxic levels as observed in conventional chemotherapy. Different classes of materials have been explored in the past two decades to develop such controlled drug delivery vehicles, which are biocompatible and effective in nature [5–10]. Liquid-crystalline folate nanocarriers represent such class of novel materials.

Folic acid is a natural vitamin B. Being a part of dietary supplements, it is biocompatible in nature. It has been reported that higher amount of folic acid does not cause any harm as it is excreted out through the urine [11]. Thus, there is lower risk of toxicity and side effects. The daily requirement of folate in the body for an average adult is reported to be 400–500 mcg/day to support

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various functions, like cell growth, DNA methylation [12,13]. Cancer cells, in general overexpress folate receptors on their surface [14–16]. This characteristic property has been used by researchers in the past, to direct nanoparticles towards tumors by attaching folic acid as a ligand on the surface of nanoparticles [17–19].

Liquid-crystalline folate nanoparticles, being composed of ordered structure, offers high encapsulation of drugs. As these nanoparticles are formed of folic acid, they will be biocompatible and non-toxic in nature. We also hypothesize that they do not require any extra step of targeting using folate ligands. Moreover, in chemotherapy, drugs are administered along with a supplement of folic acid to help the growth of healthy cells, which are targeted by the toxic effects of the drug [2,3]. As these nanoparticles disintegrate, both folic acid and encapsulated drug will be released into the medium. The release of folic acid along with the methotrexate will automatically help the growth of healthy cells off-targeted by the nanoparticles, if any. Therefore, folic acid provides strong motivation to be explored as a nanocarrier for controlled chemotherapy.

Folic acid exhibits liquid-crystalline behavior in aqueous solution by forming a self-assembly in the form of ordered stacks and columns [20–22]. The nanoparticles of liquid-crystalline folates exhibit highly ordered structures with the potential to encapsulate drugs along with the controlled release of those drugs. Our previous studies [23,24] have shown that folate self-assembles even at low concentration of 0.1 wt % and, we have reported a method to engineer folate nanoparticles from liquid-crystalline folates [25]. Moreover, an extensive study on controlled release of methotrexate from liquid-crystalline folate nanoparticles has been reported recently by our group [26]. This study has shown that the release rates of methotrexate can be controlled by controlling the size of nanoparticles, cross-linking cation and cross-linking concentration.

The present study reports the *in vitro* cytotoxic and cellular uptake assessment of methotrexate loaded folate nanoparticles on cultured HeLa cells. The role of release rate controlling parameters on cell survival was studied and cytotoxicity of MTX-folate nanoparticle over free MTX was compared. This study also shows the potential of folate nanoparticles in distinguishing cancer cells from normal cells through co-culture method [27]. The influence of methotrexate release rates on cytotoxicity of HeLa cells was studied. A comparative cellular uptake study was performed on folate receptor positive and negative cancer cells, which show that the mechanism of uptake via folate receptor-mediated endocytosis.

Dynamic light scattering (DLS) and scanning electron microscope (SEM) techniques are used to characterize the nanoparticles developed while the concentration of released methotrexate is determined by a method developed by high performance liquid chromatography technique. The cytotoxicity studies were carried using MTT assay while the cellular uptake was studied through fluorescence microscopy.

## 2. Materials and methods

### 2.1. Materials

Folic acid (molecular formula: $C_{19}H_{19}N_7O_6$; molecular weight: 441.3974 g/mol; PubChem:CID 6037) and hydroxy propyl methyl cellulose (HPMC) (molecular formula: $C_{12}H_{20}O_{10}$; molecular weight: 324.2848 g/mol) were purchased from Central Drug House (CDH), New Delhi. Methotrexate (molecular formula: $C_{20}H_{22}N_8O_5$; molecular weight: 454.44 g/mol; PubChem: CID 126941) was purchased from TCI Chemicals India Ltd, New Delhi. Methotrexate is an anti-metabolite and anti-folate drug, which is widely used in the majority of cancer types during conventional chemotherapy [28]. Fig. 1a–c shows the chemical structure of folic acid, methotexate and HPMC

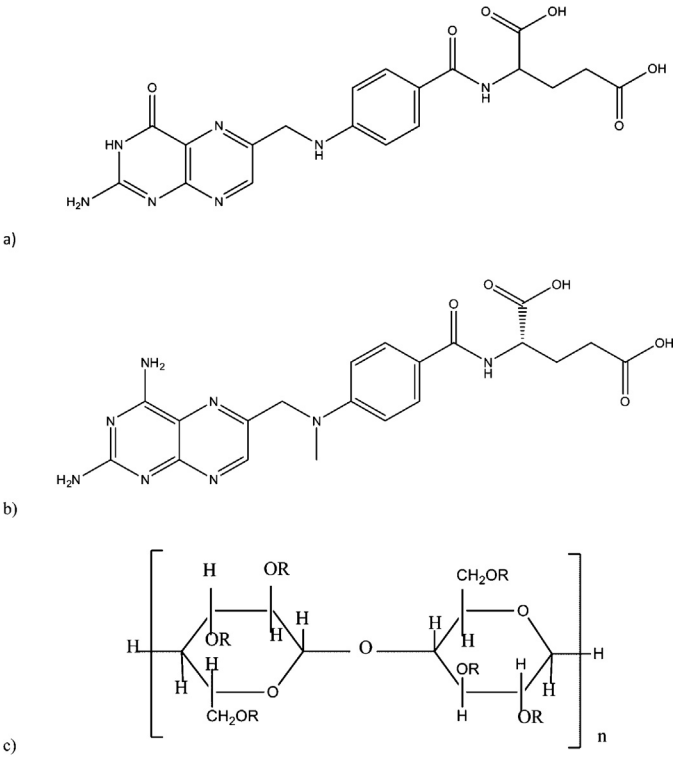


**Fig. 1.** Chemical structure of (a) folic acid, (b) methotrexate (MTX), (c) hydroxy propyl methyl cellulose (HPMC).

respectively. Normal saline (0.8% NaCl solution) was used as a release medium for *in vitro* release studies of methotrexate. (All concentrations reported in this study are in weight/weight basis).

### 2.2. Preparation of liquid-crystalline folate solution

Folic acid by itself does not dissolve in water, however, in the presence of NaOH, it forms the liquid-crystalline solutions. It has been reported in the past that folic acid molecules get completely ionized by NaOH and can be dissolved in water easily. Liquid-crystalline behavior is observed between the pH values 6.5–7.5 [23]. The stock solution of folic acid was neutralized by adding 1 N NaOH solution dropwise till the solution turned liquid-crystalline (visually) while ensuring that the pH was less than 7.0.

### 2.3. Preparation of methotrexate encapsulated folate nanoparticles

Folate nanoparticles can be engineered using HPMC as a tool. HPMC is a water-soluble cellulosic biocompatible polymer [29], which is used in the food industry as additives, emulsifiers, thickening and suspending agents as well as in pharmaceutical industry.

Due to difference in the nature of interactions of folic acid and HPMC in aqueous solution, folate nanoparticles are formed. In aqueous state, folate forms two-phase system with HPMC as folate ions with aromatic rings prefer to interact with themselves, rather than with HPMC. Liquid-crystalline folates when mixed with HPMC get dispersed into nano-domains; which on cross-linking with multivalent salts forms stable nanoparticles. We have extensively studied the designing of folate nanoparticles from liquid-crystalline folate solutions in terms of phase behavior, size distribution and thermodynamics aspects previously [25]. It has been reported by us that the size of the folate nanoparticles can be controlled by the choice of folate or HPMC concentration, with