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In vitro controlled release of Rifampicin through liquid-crystalline folate nanoparticles

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ABSTRACT

Rifampicin is one of the frontline drugs for tuberculosis therapy but poor bioavailability of Rifampicin in combination with other anti-tuberculosis drugs is a subject of concern. Nano-based formulations for sustained release of anti-tubercular drugs have been shown to increase antibacterial efficacy and pharmacokinetic behavior. In the present study, liquid-crystalline folate nanoparticles were designed for sustained delivery of Rifampicin and its *in vitro* release study is reported. Liquid-crystalline nanoparticles of biocompatible folate ions consist of self assembled structures, resulting in high encapsulation, controlled release and low drug losses of about 20-30%, which is significant in itself. This study reports the size-control method of forming Rifampicin encapsulated folate nanoparticles as well as the parameters to control the release profiles of Rifampicin through these nanoparticles. These designs are able to present sustained release for over 25 days. The effect of different parameters such as nanoparticles size, type of cross-linking cation, cross-linking cation concentration and drug-loading on Rifampicin release was studied *in vitro*. The intracellular uptake and low cytotoxicity of nanoparticles by alveolar macrophages was also demonstrated using fluorescence microscopy and MTT assay respectively.

1. Introduction

Tuberculosis is a highly contagious and persistent bacterial infection caused by *Mycobacterium tuberculosis*. Rifampicin is one of the four drugs along with Isoniazid, Pyrazinamide and Ethambutol used in combination therapy to treat tuberculosis. Rifampicin inhibits the bacterial RNA synthesis by binding to the beta-subunit of bacterial DNA dependent RNA polymerase (DDRP). Inhibition of DDRP leads to blocking of the initiation chain formation in RNA synthesis. It is one of the most effective anti-tuberculosis agents available and is a frontline drug for the treatment of tuberculosis. However, poor bioavailability of Rifampicin in combination with the other three drugs is a subject of concern. This poor bioavailability leads to sub-therapeutic levels of Rifampicin in the body which increases the risk of developing multiple drug resistance tuberculosis (MDR-TB) [1].

There is a significant potential advantage of using nanoparticles as a drug delivery medium in tuberculosis. When administered intravenously, the nanoparticles in the size range of 100–400 nm follow the route of other foreign particles and get endocytosed by resident macrophages and monocytes. On the other hand, in case of infection caused by intracellular persisting microbes (like *M. tuberculosis*); macrophages become reservoirs for pathogens, thus representing targets for delivery of antimicrobial agents. Hence, nanoparticles improve drug delivery to macrophages, increasing the amount of drug reaching the target site, which allows the reduction of overall therapeutic dose and decrease of side-effects [2–5].

A sizeable number of studies have been done to show the effectiveness of sustained release of anti-tubercular drugs through various nanoparticles both *in vivo* and *in vitro* [6–9]. These studies have been summarized in supplementary information.

Folic acid is a chromonic molecule which self-assembles to form liquid-crystalline solution. Our previous studies have shown that folic acid self assembles in the form stacks even at low concentrations of 0.1 wt % [10]. It represents a class of materials that exhibit high-order self-assembly behavior which is driven primarily by enthalpic interactions. Chromonics are structurally composed of aromatic rings with hydrophilic groups at the periphery and show liquid crystalline behavior in aqueous solutions.

Folic acid derivatives are observed to behave like chromonic molecules and exhibit liquid crystalline behavior. Folic acid is a natural vitamin which is ingested by humans as part of their daily diet. It is found in green leafy vegetables, dried beans, peas and citrus fruits. It is essential for the formation of new cells and DNA in our body. Thus, using folic acid as a drug delivery carrier has a lower risk of toxicity and side effects [11].

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Folate nanoparticles can be engineered from liquid crystalline folate solution by mixing it with HPMC (Hydroxypropyl Methylcellulose) and their size can be controlled by varying the relative concentrations of HPMC and folic acid [12]. Hydroxypropyl Methylcellulose (HPMC) is a non-toxic, semi-synthetic, inert; viscoelastic polymer used as a food additive, as well as an excipient and controlled-delivery component in oral medicaments [13]. Its structure is shown in Fig. S1c. 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 forming a two-phase system.

When folate is mixed with HPMC for a sufficient amount of time nano domains of size 100–400 nm are formed [12]. These domains are cross-linked with multivalent salts like Calcium Chloride or Zinc Chloride to form stable nanoparticles. During the cross-linking process, multivalent ions (Ca^{2+} or Zn^{2+}) exchange with the monovalent cation (Na^{+}) in the folate solution. When suspended in release medium containing monovalent salts (saline, phosphate buffered saline *etc.*) the folate assembly is disrupted leading to release of folic acid and the encapsulated drug. The rate of disruption is influenced by mass transfer of the monovalent cation to the particle surface as well as the exchange kinetics of the monovalent ion with the multivalent ion.

The advantage of using liquid crystalline folate nanoparticles as a drug delivery medium is that folate forms highly ordered structures which lead to comparatively low drug losses as compared to other drug delivery mediums. Rifampicin is encapsulated in this ordered structure through intercalation within the folate stacks which implies that it participates in the folate self-assembly. This ordered structure is present in the nanoparticles developed. The nanoparticles are cross-linked with multivalent cation to keep them stable. The drug molecules can be released in a controlled manner by disrupting this folate assembly.

This paper presents folate nanoparticles as a novel material for sustained release of Rifampicin, an anti-tubercular drug, in the treatment of tuberculosis. The present study discusses the method to encapsulate Rifampicin in folate nanoparticles and understand the parameters by which Rifampicin can be released in a controlled manner through these nanoparticles under different conditions. Dynamic Light Scattering (DLS) and Scanning Electron Microscope (SEM) techniques are used to characterize the nanoparticles developed while the concentration of released Rifampicin is determined by a measuring the absorbance value with UV-visible spectrophotometer. This study also addresses the cellular uptake and cytotoxicity of folate nanoparticles by alveolar macrophages through fluorescence microscopy and MTT assay respectively.

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 HPMC (Hydroxypropyl Methylcellulose) (molecular formula: $C_{12}H_{20}O_{10}$; molecular weight: 324.2848 g/mol) were purchased from Central Drug House (CDH) New Delhi. An aqueous stock solution of 5 wt% folic acid and 10 wt% HPMC was prepared using de-ionized water. Folic acid does not dissolve in water by itself; however in the presence of sodium hydroxide it forms the liquid crystalline solutions. The stock solution of folic acid was neutralized by adding 1 M NaOH solution drop-wise till the solution turned liquid crystalline (visually) while ensuring that the pH was less than 7.0. It has been reported in 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 and 7.5 [14]. Rifampicin (PubChem: CID 5381226) was purchased TCI Chemicals, India. Fig. S1b shows the chemical structure of Rifampicin. Normal saline (0.8% NaCl solution) was used as a release medium in all the experiments to perform the release studies.

2.2. Encapsulation of Rifampicin in liquid-crystalline folate assembly

Rifampicin is added to liquid-crystalline folate solution at loading amount of 10% and 30% of folic acid concentration. This solution is continuously stirred for 1 h at 300 rpm. To study the effect of Rifampicin on folate self-assembly, X-Ray diffraction was performed with the 5% folate solution loaded with 0.5% and 1.5% Rifampicin.

2.3. Preparation of Rifampicin encapsulated folate nanoparticles

The desirable size range of the nanoparticles in tuberculosis drug delivery has been reported to be 100–400 nm [15]. Rifampicin encapsulated folate nanoparticles were prepared in this size range by changing the relative concentrations of folate and HPMC. 5% liquid crystalline folate solution was mixed with 1%, 5% and 10% HPMC in the ratios (w/w) of folic acid to HPMC. This mixture of folate solution and HPMC was mixed by continuous stirring at 800–900 rpm for 6 h at room temperature.

2.4. Cross-linking of nanoparticles

Multivalent cationic salts (aqueous solutions of 10% $CaCl_2$ and $ZnCl_2$) in the weight ratio 1:10 (folate–HPMC mixture: salt solution) were used to form cross-linked folate nanoparticles. Mixing was performed with continuous stirring at 800–900 rpm for 8 h at room temperature. This resultant solution was centrifuged (Eppendorf centrifuge 5810R) at 10,000 rpm for 10 min. The obtained pellet is suspended and washed with de-ionized water and centrifuged again at 10,000 rpm for 10 min. This pellet is re-suspended in de-ionized water and 1 ml of this suspension was sonicated (Metrex ultrasonic bath sonicator) with 5 ml of de-ionized water for 1 min at room temperature (35° C). This sonicated mixture is used for size distribution studies by DLS (Dynamic Light Scattering) technique. Further, the pellet obtained after centrifugation was lyophilized (Christ Alpha 1-4 LD Plus Freeze Dryer) at −49 °C and 0.002 mbar vacuum pressure to remove any HPMC present.

2.5. Encapsulation efficiency of Rifampicin

In each experiment, mass balance of Rifampicin was carried out to determine the amount of drug encapsulated in the folate nanoparticle. To separate the HPMC and salts that were not bound, a centrifugation step was performed after cross-linking the nanodomains with multivalent salts. During this step, drug loss was calculated by analyzing the drug concentration in the supernatant with the help of a UV–vis spectrophotometry. Accounting for the losses during the method of preparation, encapsulation efficiency was calculated by the given formula:

$$\text{Encapsulation efficency} = \frac{\text{Initial amount of drug added} - \text{Amount of drug lost during processing}}{\text{Initial amount of added}} \times 100$$
$$\text{Encapsulation efficency (\%)} = \frac{\text{Amount of drug in pellet}}{\text{Initial amount of drug loaded}} \times 100 \quad (1)$$

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