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Steady-state fluorescence study on release of camptothecin from agar hydrogel

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Abstract

The slow drug release processes from agar hydrogel were studied by steady-state fluorescence (SSF) measurement. An anticancer drug, camptothecin (CPT), was used as the release drug and the fluorescence probe for the real-time monitoring of the release processes by measuring the fluorescence intensity of the probe. The release kinetics of CPT at different temperatures was investigated. The Fickian diffusion model was adopted to fit the results and a good linear relationship could be observed between the logarithmic release fraction and the releasing time. The diffusion coefficients (D) at these conditions were thus obtained from the slopes of the fitting curves. By plotting D against temperature, the diffusion coefficient of CPT was found to obey the Arrhenius relation and the activation energy was then obtained to be 70.6 kJ mol^{-1}.
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1. Introduction

Fluorescence techniques, such as steady-state spectroscopy, fluorescence anisotropy and fluorescence decay measurements, are powerful tools for studies of molecular diffusion or molecular interactions. Due to the high sensitivity, versatility of information and commercially available instrumentation, fluorescence techniques have been extensively applied in many areas such as analytical chemistry, biochemistry, cell biology, environmental science, etc. (Winnik, 1986; Lakowicz, 1999).

Recently, a few reports have been found on the application of fluorescence techniques to study of polymeric gels. Various fluorescence techniques were used to study the gelation, gel swelling or slow release of drugs from chemical or physical gels. A fluorescence probe study was carried out by Hu et al. (1992, 1993) for a gel system. They have successfully applied the flu-

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orescence depolarization technique to investigate the volume phase transition of the acrylamide gel induced by the change in solvent composition and pH. The time-resolved and steady-state fluorescence techniques (SSF) were employed to study isotactic polystyrene in its gel state where the excimer spectra were determined to monitor the existence of two different conformations in the gel state of polystyrene (Wandelt et al., 1991). The sol–gel transitions in a free radical cross-linking copolymerization process was in situ observed by Pekcan et al. by using the SSF technique (Pekcan et al., 1994, 1996a,b; Yilmaz et al., 1998a). The same technique was also applied for studying the swelling of disc-shaped poly(methyl methacrylate) gels made with different cross-linking densities (Yilmaz et al., 1998b), or with different toluene contents (Pekcan et al., 1997). The slow release processes were studied in these gels in various solvents (Pekcan et al., 2002). Pyrene was used as a fluorescence probe in all these experiments and its SSF emission intensity was recorded to monitor the above processes. A single photon counting method in conjunction with direct energy transfer was used to study the diffusion of a dye labeled poly(methyl methacarylate) sterically stabilized by polyisobutylene, where the mean lifetimes of fluorescing donor molecules were measured to monitor diffusion (Pekcan, 1993, 1996).

Polysaccharide hydrogels have attracted biomedical researchers due to their good biocompatibility and nature origin (Matin et al., 1992; Chen et al., 1995). Hydrogels containing specific sugars may lead to improved adhesion and growth of microbial and mammalian cells (Dordick et al., 1994). Agars are cell-wall polysaccharides extracted with water from certain members of marine red algae and mainly composed of alternating β-(1-4)-D-galactose and α-(1-3)-3,6-anhydro-L-galactose repeating units (Araki and Hirase, 1953). They are widely used as gel-forming agents, thickeners, water-holding agents and stabilizers in the food industry. The gelling ability of agar is associated not only with the chemical characteristics of the molecule but also with the molecular weight (Fuse and Goto, 1971; Mitchell, 1980; Mouradi-Givernaud et al., 1992).

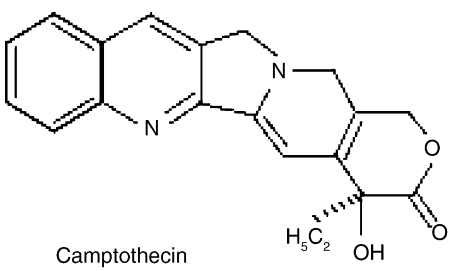
Camptothecin (CPT) is an anticancer drug, which is a plant alkaloid and very effective in the treatment of gastric, rectum and bladder tumors (Brezova et al., 2003). It is a pentacyclic alkaloid isolated and characterized by Wall et al. (1966) from camptotheca acuminata, which was shown to exhibit potent cytotoxic activity against a range of tumor cell lines. More information about CPT can be found from the review by Pizzolato and Saltz (2003).

The main objective of this work is to use an anticancer drug (CPT) both as a drug and as a fluorescent probe to study its release behavior from an agar hydrogel. Our study focuses on the release mechanism of CPT and the effect of release condition. As a fluorescent dye, CPT is easy to be detected by a fluorescence technique such as SSF spectrometry. In this work, the SSF experiments were performed for a real-time monitoring of the slow release processes. The release diffusion coefficients, D, at various temperatures were then measured. The activation energy was also obtained by using the Arrhenius relation equation. The slow release mechanisms will be discussed. The knowledge produced from this study will be helpful for understanding of the release mechanism of CPT from hydrogels and useful for pharmaceutical design of release devices.

2. Materials and methods

2.1. Materials

Agar ($(C_{12}H_{18}O_9)_x$, $M_w = 3000–9000$) and CPT ($C_{20}H_{16}N_2O_4$, $M_w = 348.4$) were purchased from Sigma–Aldrich company and used directly without further purification. Deionized (DI) water was obtained from the Millipore purification system (Alpha-Q: CPMQ004R1). The chemical structure of CPT is given in Scheme 1.



Camptothecin

Scheme 1. Chemical structure of camptothecin.

2.2. Hydrogel preparation

A cylinder-shaped hydrogel of agar (1 g agar/19 g DI water), which contained 3.48 mg CPT (0.01 mmol), was prepared. The inner diameter of the cylinder was 22.80 ± 0.02 mm. CPT was loaded by physically mixing it with the agar gelling solution. This method of drug loading is commonly employed in drug delivery studies (Andreopoulos et al., 2001; Guerra et al., 2001). The agar was first dissolved by extensive stirring in DI water at 95 °C until an optically clear solution was obtained. The temperature was subsequently reduced to 60 °C and held at 60 °C for 1 h (Lead et al., 2003). The resultant solution was slowly poured into a pre-heated (60 °C) cylindrical mold to allow a natural cooling of the solution to room temperature (25 °C). The solution gelled at around 36 °C. Identical disc-shaped release samples (thickness = 2.5 mm) were cut from the cylindrical gel for the subsequent release experiments at different temperatures.

2.3. Release experiments

The release of CPT from the agar hydrogel into DI water was performed at different temperatures. In all experiments, the identical disc-shaped hydrogel samples (volume ∼ 1 cm^3) were used. At each temperature (23, 30, 37 or 44 °C), the sample was totally immersed in a bottle containing 50 ml of DI water so that a perfect sink condition can be assured (Ritger and Peppas, 1987). At certain time intervals, 3 ml solution was taken out from each release system (i.e. the surrounding releasing medium) for SSF measurements and the same volume of a fresh DI water, which was previously kept at the same temperature as the individual release system, was added in order to maintain a constant volume of the surrounding solution.

2.4. Steady-state fluorescence measurements

SSF measurement is the most commonly used fluorescence technique, where a sample is illuminated with a monochromatic UV light and the intensity or emission spectrum is recorded. The typical fluorescence emission spectrum of CPT aqueous solution can be observed in Fig. 1. The peak intensity is around 440 nm.

SSF measurements for the surrounding solutions in the above-mentioned release experiments were carried out using a Shimadzu-RF5301PC spectrofluorimeter equipped with a jacketed cuvette holder. All measurements were made at the front-surfaces of the samples at 45° position with an excitation wavelength of 370 nm and the slit widths for excitation and emission were both kept at 1.5 mm. The emission intensities at 440 nm were recorded for all the solutions measured.

3. Results and discussion

3.1. Release profiles determined by SSF spectra

At certain time intervals, the SSF emission spectra for the surrounding solutions at four temperatures (23, 30, 37 and 44 °C) were recorded. Fig. 1 shows the change of the SSF spectra as the releasing time increases, which was obtained from the release experiment at 37 °C and represents the typical change of the SSF emission spectra occurring as a function of time in any of the release experiments. As shown in Fig. 1, the SSF emission intensity (I_t) of the surrounding solution increases with release time. Since I_t is immediately related to the amount of the probe in the solution, the feature of the intensity curves in Fig. 1 may suggest that the longer the release time, the more the CPT molecules have been released to the surrounding solution.

The different release behaviors under four temperatures are illustrated by the difference in their SSF spectra intensities at a given release time as shown in Fig. 2,

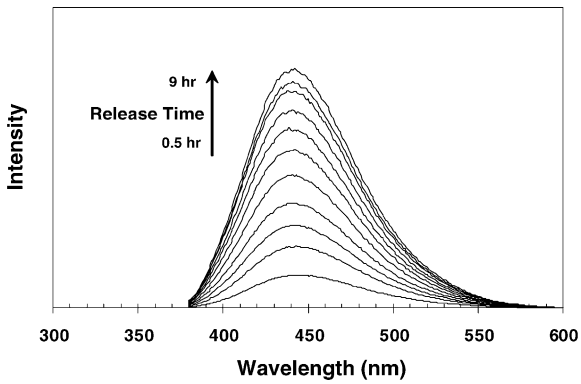


Fig. 1. Fluorescence emission spectra of the surrounding solution in the release experiment of CPT from the agar gel. The excitation wavelength was 370 nm. The measurements were made at a time interval of 0.5 h initially and 1 h later. The lowest and highest curves were obtained with the release time of 0.5 and 9 h, respectively.

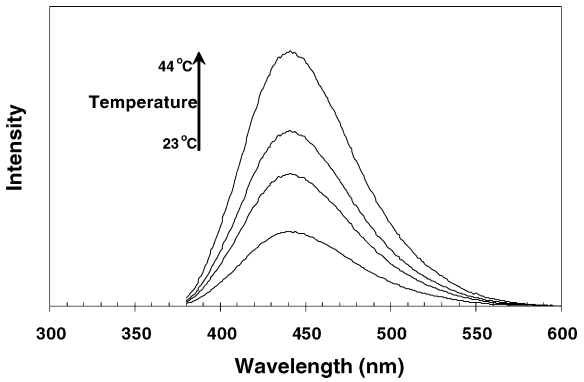


Fig. 2. Fluorescence emission spectra of the surrounding solutions in the release experiments of CPT from the agar gel at 23, 30, 37 and 44 °C. The excitation was done at 370 nm and the release time was 8 h.

where the release time was 8 h. The similar profile curves could be observed as a function of time except at the very beginning time regions. It is apparent that a higher temperature led to a faster release of CPT into the surrounding solution.

3.2. Relationship between M_t/M_∞ and I_t/I_∞

As found from literature, the fraction (M_t/M_∞) of the amount of drug released at time t has been widely employed to depict the drug release behavior. Here, M_t is the amount of drug released at time t, M_∞ is the amount of drug released at infinite long time. In the present work, only I_t, the emission intensity of the surrounding solution at time t, and I_∞, the emission intensity of the surrounding solution when the release reached an equilibrium (i.e. the intensity approached to a constant value), could be obtained by SSF measurements. Within the concentration range ($<1.2 \times 10^{-5}$ M) of CPT where I_t obeys the Beer's law, M_t and M_∞ are proportional to I_t and I_∞, respectively (Yilmaz et al., 1998b; Pekcan et al., 2002). Therefore, the fraction (M_t/M_∞) of the released drug can be simply expressed as:

$$\frac{M_t}{M_\infty} = \frac{I_t}{I_\infty} \quad (1)$$

In this work, we use this relation to determine the release behavior of CPT from the hydrogel.

3.3. Profiles of M_t/M_∞ versus time

The slow release kinetics of a substance in physical or chemical gels is of scientific significance and applied importance. In order to interpret the kinetics of CPT's release from the agar hydrogel at different temperatures, the fraction (M_t/M_∞) of the amount of CPT released at time t was plotted in Fig. 3 as a function of time. All experiments showed a fast release of the drug within the first 20 h; afterwards, the release slowed down, and finally tended to level off. It can be clearly seen that the release of CPT increased with increasing temperature. As expected, the sample at 23 °C displayed a prolonged release compared to that at a higher temperature. In these release processes, the drug diffused out from the hydrogel by the driving force of the concentration gradient between the internal hydrogel and the surrounding solution, the increase in temperature obviously enhanced the activity of CPT molecules and therefore promoted a faster release of drug from the hydrogel. The other reason for the enhanced release would be the thermal expansion of the hydrogel, which may result in a looser network to allow the drug molecules to be released faster.

The model-independent parameters (Bonferoni et al., 1998) were obtained from the individual experimental curves and summarized in Table 1, where 2h%, $t_{50\%}$ and t_∞ represent the percentage release after 2 h of dissolution, the time at which 50% amount of the drug was released and the time to achieve an equilibrium

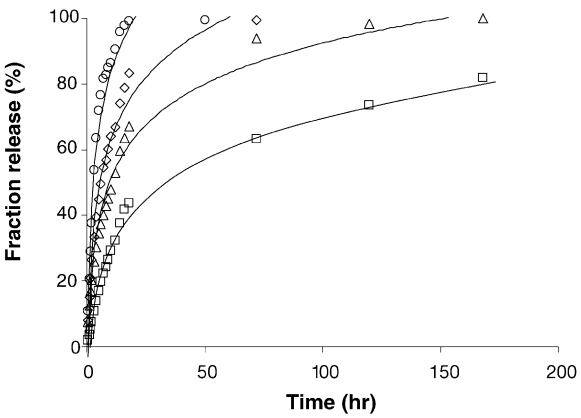


Fig. 3. Fraction release, M_t/M_∞, of CPT from agar hydrogel at different temperatures as a function of time t (◯, 44 °C; ◊, 37 °C; △, 30 °C; □, 23 °C).

Table 1
Release of CPT from agar hydrogel: experimental designation, release parameters and diffusion coefficients

Sample	Temperature (°C)	$t_{50\%}$ (h)	2h%	t_∞	D (cm^2 s^{-1})
#1	23	31.50	13.48	1.5 weeks	5.04×10^{-8}
#2	30	10.09	20.39	1 week	1.09×10^{-7}
#3	37	6.02	26.47	6 days	1.60×10^{-7}
#4	44	3.25	36.67	2 days	3.67×10^{-7}

release, respectively. From Table 1, the temperature effect on the release behavior can be clearly observed. At the highest temperature (44 °C) used in this work, it only took 3.2 h to achieve 50% release and 2 days to achieve the equilibrium, while the time needed at the lowest temperature (23 °C) to achieve the same levels of release was 31.5 h and 1.5 weeks, respectively.

3.4. Diffusion coefficients

The release of an active substance from hydrogels is classically assumed to take place by diffusion (Muhr and Blanshard, 1982). Diffusion coefficient thus appears as a key parameter if a device has been designed to release a solute at a pre-determined rate. Diffusion of drug or protein is well described by the Fick's equations (Peppas, 1986), which correlate the solute fluxion with its chemical potential gradient in the system. Thus, the structure and pore size of the gel, the polymer composition, the water content and the nature and size of the solute are all taken into account of the diffusion coefficient of the solute. The Fick's second law describes the solute concentration variation in a flat gel sample (C) as a function of time (t) and distance (x) (Crank, 1975):

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} \tag{2}$$

where the diffusion coefficient D is assumed to be constant and the boundary conditions are:

$$t = 0, \quad -\tfrac{1}{2}l < x < \tfrac{1}{2}l, \quad C = C_1$$
$$t > 0, \quad x = \pm\tfrac{1}{2}l, \quad C = C_0$$

where l is the thickness of the flat sample.

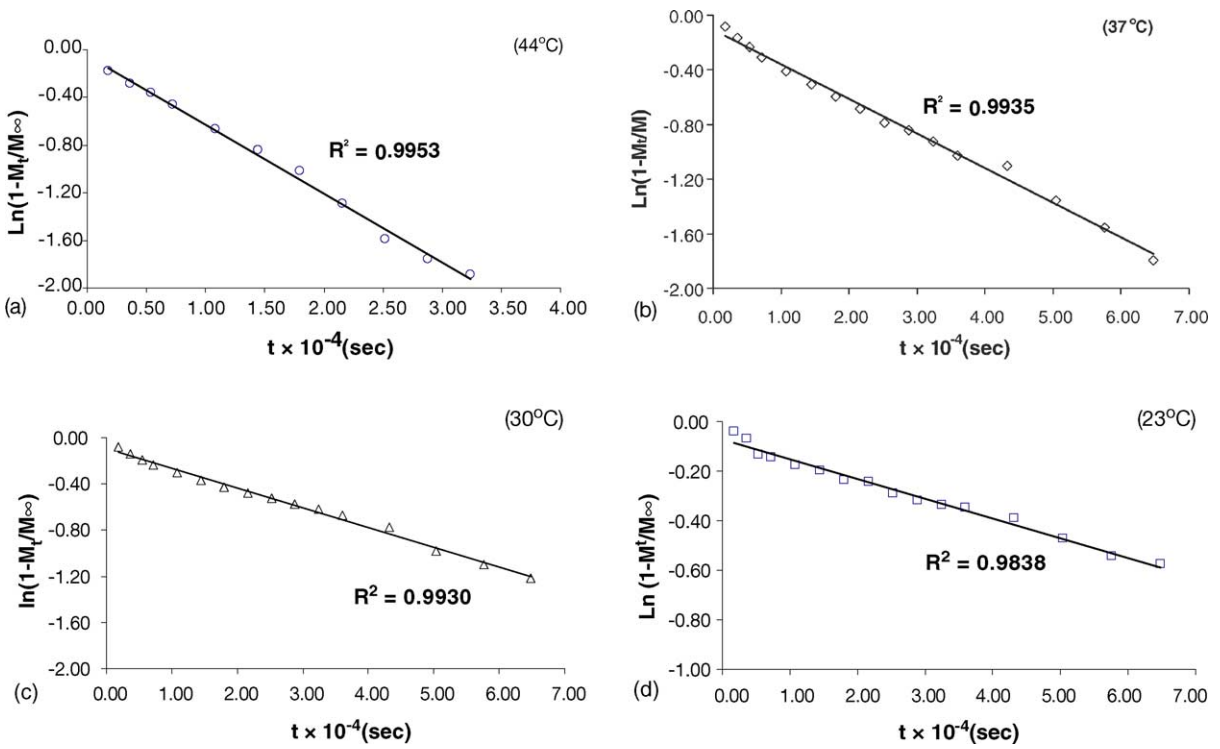


Fig. 4. (a–d) Semi-logarithmic plots of the data in Fig. 3 as a function of time t at different temperatures (◯, 44 °C; ◊, 37 °C; △, 30 °C, □, 23 °C).

The solution to the Fick's law in the form of a trigonometric series under the above-specified boundary conditions is:

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(n+1)^2}\exp\left\{-\frac{(2n+1)^2 D\pi^2}{l^2}t\right\} \quad (3)$$

By taking only the first term in the summation (Σ) series and performing a logarithmic transformation, Eq. (3) can be simplified to Eq. (4):

$$\ln\left(1 - \frac{M_t}{M_\infty}\right) = \ln\frac{8}{\pi^2} - \frac{D\pi^2}{l^2}t \quad (4)$$

By plotting $\ln(1 - (M_t/M_\infty))$ against t, D can be determined from the slope, $-D\pi^2/l^2$, of a linear regression of Eq. (4). This method has been extensively adopted to obtain diffusion coefficients from drug release data (Yilmaz et al., 1998b; Pekcan et al., 2002; Han et al., 2000).

The release diffusion coefficients now can be obtained for CPT molecules in the agar gel by fitting the data in Fig. 3 using Eq. (4). The results are shown in Fig. 4, where a linear relationship can be observed except at long times which correspond to a saturation state of the slow release process. Using Eq. (4) for the data in Fig. 4, D values are obtained and are listed in Table 1. As expected, D values at lower temperatures are found to be smaller than those at higher temperatures.

3.5. Activation energy

As seen in Table 1, the D value increases as temperature increases, indicating that a relationship between D and T may exist. The Arrhenius equation was therefore examined for the D values (Erdoğan et al., 2001):

$$D = D_0 \exp\left(-\frac{E_a}{RT}\right) \quad (5)$$

where E_a is the activation energy, R the gas constant ($R = 8.31\ \mathrm{J\,mol\,K^{-1}}$), T the absolute temperature, and D_0 is the diffusion coefficient at $T = \infty$. The logarithmic form of the D data is plotted against T^{-1} in Fig. 5, where the slope of the linear relation produces the activation energy E_a to be $70.6\ \mathrm{kJ\,mol^{-1}}$ for the release of CPT from the agar hydrogel.

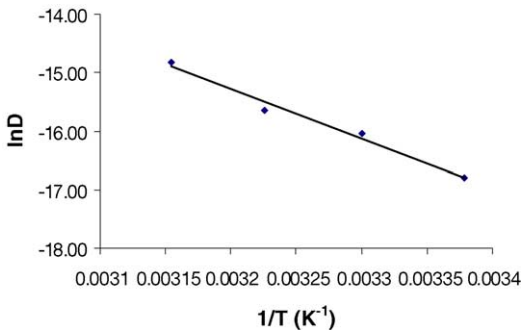


Fig. 5. The Arrhenius plot of the diffusion coefficients of CPT in the agar gel.

4. Conclusions

The results in this work have shown that the steady-state fluorescence method can be used for real-time monitoring of the slow release processes. In this method, in situ fluorescence experiments have been proved again to be an easy and powerful tool for studying a slow release process. The release of CPT from the agar hydrogel at different temperatures was investigated using the SSF method. The diffusion coefficients were obtained by fitting the release result to the Fickian diffusion model, which were found to be in the range from 5.04×10^{-8} to $3.67 \times 10^{-7}\ \mathrm{m^2\,s^{-1}}$. The D values related to temperature in an Arrhenius relation and the activation energy was obtained to be $70.6\ \mathrm{kJ\,mol^{-1}}$. The agar hydrogel has shown to be one of the suitable systems for the release of CPT.

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