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Investigating the Fluorescence Quenching of Doxorubicin in Folic Acid Solutions and Its Relation to Ligand-Targeted Nanocarriers

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Folic acid (FA) is one of the most utilized moieties in active (ligand) drug delivery. The folate receptor is widely expressed on the surface of several cell lines and tumors; including ovarian, brain, kidney, breast, and lung cancers. During our previous experiments with Doxorubicin (Dox) encapsulated in folate-targeted micelles, we found that flow cytometry underestimated the amount of drug that accumulates inside cells. We attributed this effect to the quenching of Dox by FA and herein investigate this phenomenon in an attempt to obtain a correction factor that could be applied to the fluorescence of Dox in the presence of FA. Initially, we examine the effect of pH on the fluorescence spectra of FA, Dox, equimolar solutions of FA and Dox in water, HCl (0.1 M), and NaOH (0.1 M) solutions. We then measure the effect of the gradual increase of FA concentration on the fluorescence intensity of Dox in phosphate-buffered saline (PBS) solutions (pH of 7.4). Using the Stern-Volmer equation, we estimate the association constant of FA-Dox to be $K_{sv} = 1.5 \times 10^4$ M^{-1}. Such an association constant indicates that at the concentrations of FA used in targeted drug delivery systems, a significant concentration of Dox exists as FA-Dox complexes with a quenched fluorescence. Therefore, we conclude that when Dox is used in FA-active drug delivery systems, a correction factor is needed to predict the correct fluorescence intensity of agent *in vitro* and *in vivo*.

Keywords: Folic Acid, Fluorescence, Quenching, Doxorubicin, Drug Delivery.

1. INTRODUCTION

Recently the pharmaceutical industry has directed considerable attention to developing new drug delivery systems that are able to selectively transport therapeutic agents into desired locations safely and in a concentration-dependent manner. Studies using nanocarriers (including liposomes and micelles) have shown promising results in this area, as these nanoparticles with simple, yet effective structures, showed high efficiency as drug delivery vehicles (both *in vitro* and *in vivo*). When considering nanocarriers in drug delivery systems (DDS), there are three main targeting techniques to be considered, namely: passive, triggered and active (ligand) targeting. Studies have shown that DDS can be significantly enhanced if a multimodal-targeted vehicle (using all three targeting techniques) is utilized.[1]

Tumors have leaky capillaries with smaller diameters compared to healthy tissue. The presence of defective vasculature in malignant neoplasms (also known as the enhanced retention and permeation (EPR) effect) enhances the accumulation of drug-loaded nanocarriers and hence elevates the concentration of the anti-neoplastic agent at the diseased site.[2] The second type of targeting includes the use of a trigger to achieve delivery. Triggered targeting involves the use of internal or external stimuli to initiate drug release. These triggers include temperature, magnetic fields, ultrasound waves, light waves, electric fields, and pH.[1,3] The third form of targeting is ligand targeting (sometimes referred to as active targeting) and it refers to the labeling of the nanovehicle's surface with a targeting moiety. These decorating moieties include peptides, antibodies, hormones, aptamers and low molecular weight ligands (e.g., folate).[4,5]

In an ideal therapeutic scenario, ligands are chemically conjugated to the carrier's surface in order to target specific receptors expressed on the cell membrane of cancer cells and induce endocytosis. These small-molecule ligands are extensively used in cancer drug delivery research

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due to their ease of conjugation to the carriers (liposomes, micelles or solid nanocarriers) and to the abundance of their receptors on several cell lines and tumors. One of the most important low-molecular-weight targeting molecules is folic acid, but several carbohydrates (e.g., galactose, mannose, etc.) have also been studied.[6]

Folic acid (FA) (Scheme 1) is a water-soluble vitamin, and has several functions in cell growth and division. Its use in drug delivery depends on its strong binding affinity toward the folate receptor (FR), a receptor widely expressed on the surface ovarian, brain, kidney, breast, lung and other cancers.[6, 7]

Doxorubicin (Dox) (Scheme 1) is one of the most widely used chemotherapy anti-neoplastic agents in medicine. Previous results of FA-targeted micellar treatments with Dox have shown that cell lines overexpressing the folate receptor (FR+) demonstrate an enhanced uptake of the chemotherapeutic agent when compared to the non-targeted drug.[8] We have previously reported the synthesis of folated polymeric micelles (namely Pluronic P105 micelles) and the use of acoustic power to release their micellar contents spatially and temporally.[9, 10] While attempting to quantify the amount of Dox encapsulated inside folated polymeric micelles, we noticed that its measured fluorescence (using fluorometry and flow cytometry) is underestimated when compared to the same anti-neoplastic agent encapsulated inside non-folated micelles.[1] Upon further investigation, we concluded that FA quenches the fluorescence of Dox, as reported previously.[11, 12] Hence, in this paper, we investigate the binding affinity of FA-Dox, causing this quenching phenomenon, and make a recommendation on whether a correction factor is needed when utilizing FA as an active (ligand) targeting moiety in drug delivery of Dox.

2. MATERIALS AND METHODS

2.1. Solution Preparation

Approximately 5.0 g of folic acid (Sigma-Aldrich, Darmstadt, Germany) were dissolved in 40 mL of phosphate buffered saline (PBS) solution. The resultant mixture was vortexed for 10 minutes and then sonicated for 20 minutes using a sonicating bath (Elma D-78224, Singen, Germany) equipped with two piezoceramic transducers, driven at approximately 40 kHz, until the solution was saturated with folic acid. Aliquots of 0.25 mL of a Dox (Doxorubicin-hydrochloride, Sigma-Aldrich, Darmstadt, Germany) stock solution (200 μg/mL) were then introduced (at room temperature) into PBS solutions with different concentrations of folic acid. The final concentration of Dox in all experiments was 5.0 μg/mL. Similarly, solutions of Dox/folic acid were prepared in water, 0.1 M HCl, and 0.1 M NaOH solutions.

2.2. Instrumentation

Steady state luminescence and synchronous scan luminescence spectra were recorded using Varian Eclipse (Varian, Inc., Mulgrave, Australia) spectrofluorometer. All measurements were recorded under similar conditions with the excitation and emission slit widths adjusted to 5 and 20 nm, respectively.

2.3. Data Analysis

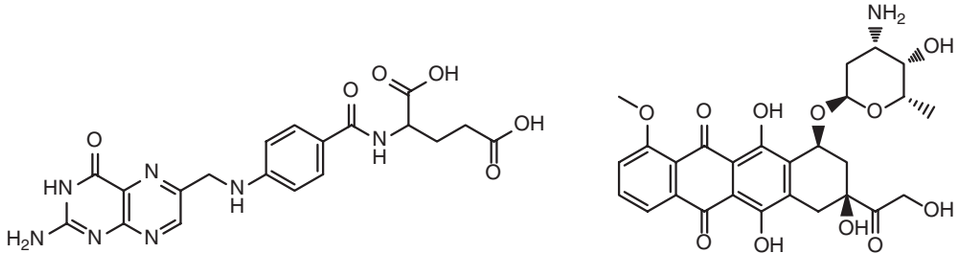
Static quenching occurs when a non-fluorescent ground state complex is formed between the fluorophore and the quencher. With the absorption of light, this complex immediately returns to the ground state without the emission of a photon. The fluorescence intensity of the fluorophore, therefore, depends on the concentration of the quencher.[13, 14] The relationship between the fluorescence intensity and the quencher concentration is given using the Stern-Volmer equation (Eq. (1)).

$$F_0/F = 1 + K_{sv}[Q] \quad (1)$$

The Stern-Volmer equation offers a direct way to obtain the association constant (K_{sv}) between the fluorophore and the quencher (Q), provided that static quenching is the only, or the dominant, quenching process.

3. RESULTS AND DISCUSSION

Steady state luminescence spectroscopy has been widely used for studying biological systems because of its simplicity, reproducibility, and low detection limits.[13] However, the use of ordinary luminescence spectroscopy



Folic acid (**FA**) Doxorubicin (**Dox**)

Scheme 1. Chemical structures of folic acid and doxorubicin.

is limited especially when the target molecules are different in their excitation and emission profiles. For example, to study the effect of FA on Dox via luminescence spectroscopy, one needs to understand the emission profile of each molecule. Figure 1 shows the emission spectra for both FA and Dox solutions prepared in PBS; FA shows a broad emission with a maximum wavelength (λ_{max}) of 466 nm upon its excitation at 400 nm whereas Dox reveals two emission bands at 562 and 594 nm upon its excitation at 488 nm. Since Dox does not have any emissions upon excitation at 400 nm, it would be difficult to obtain a detailed analysis via this technique.

Synchronous Scan Luminescence Spectroscopy (SSLS), which involves scanning the excitation and emission wavelength drives of the fluorometer simultaneously with a constant wavelength difference ($\Delta\lambda$), is very selective compared to the ordinary fluorescence methods for the analysis of fluorescing mixtures. In order to capture both the excitation and emission features of the two molecules, we utilized SSLS to monitor the behavior of Dox, FA, and mixtures of FA-Dox solutions prepared in various solvents. Figure 2 shows the SSL spectra of Dox, FA, and equimolar mixtures of FA-Dox solutions prepared in water (Fig. 2(a)), acidic (Fig. 2(b)), and basic media (Fig. 2(c)). The spectra of FA and Dox in water (Fig. 2(a)) show the characteristic SSL peaks for these compounds at 395 nm and 520 nm, respectively. The broad signal of FA with a shoulder extending to around 500 nm suggests the presence of molecular aggregation, possibly due to the low solubility of FA. The spectrum of the solution of FA and Dox, exhibits a decrease in the emission peak of Dox by ~20%, suggesting quenching of the drug's emission by FA, while the SSL peak of FA at 378 nm became narrower and higher in intensity. This change in the FA signal suggests that the presence of Dox in solution enhanced the solubility of FA and decreased the concentration of FA aggregates; such a process is a likely result of strong FA-Dox interactions.

The SSL spectra of FA and Dox in a 0.1 M HCl (Fig. 2(b)) solution shows that there is a strong quenching of FA fluorescence at 400–410 nm in the presence of Dox while there is a slight enhancement of the emission of Dox at 520 nm upon the addition of FA. These results further suggest an even stronger interaction between FA and Dox at low pH when compared to neutral pH (water). The protonation of FA in an acidic solution decreases its effectiveness as a quencher (decrease in electron density) and increases its binding affinity towards Dox possibly through hydrogen bonding and electrostatic interactions; hence, we observe a lower emission of FA and a higher emission of Dox.

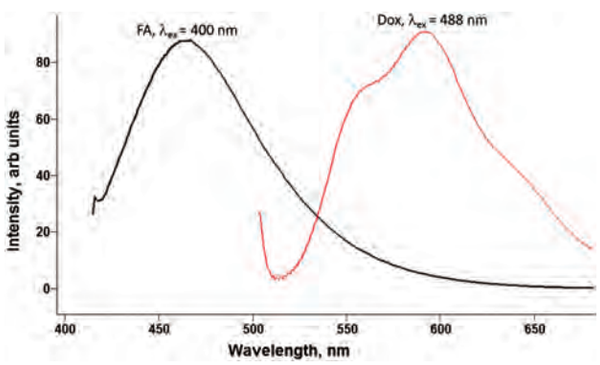


Figure 1. Emission spectra of FA and Dox aqueous solutions recorded at λ_{ex} of 400 nm and 488 nm, respectively.

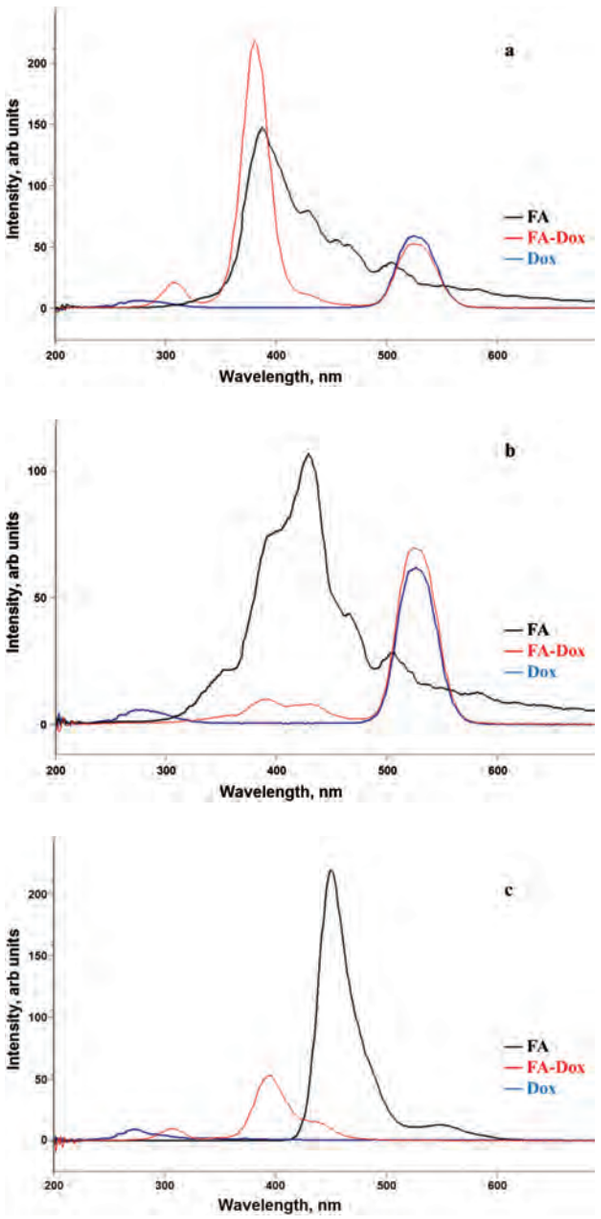


Figure 2. SSL spectra of FA (5 μg/mL), Dox (5 μg/mL), and FA-Dox monitored at $\Delta\lambda = 30$ nm in different media: (a) Water, (b) HCl solution (0.1 M), and (c) NaOH solution (0.1 M).

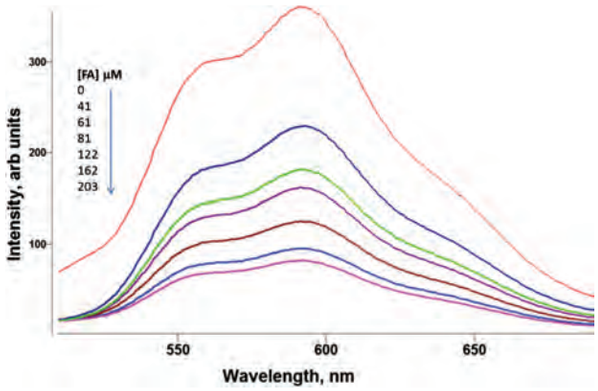


Figure 3. Emission spectra of Dox (5 μg/mL) buffered solution (pH = 7.4) recorded before and after adding various concentrations of FA.

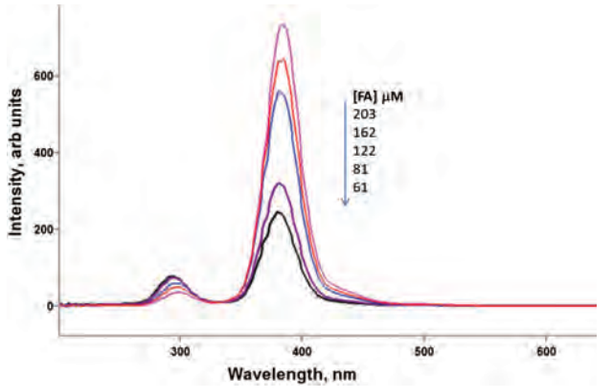


Figure 5. SSL spectra of various concentrations of FA prepared in PBS at pH 7.4.

In a basic solution (Fig. 2(c)), however, the fluorescence of FA shifts to a higher wavelength (452 nm) with a narrower emission range than that observed in acidic or neutral solutions. This result is consistent with the deprotonation of FA groups leading to better conjugation within the pteridine ring system and is in agreement with the previously reported spectra.[14] Meanwhile, Dox fluorescence is totally quenched even in the absence of FA (Fig. 2(c)), due to the deprotonation of the hydroxyl phenol groups which leads to fluorescence quenching.[17]

Since all therapeutic studies are conducted at physiological pH, we investigated the effect of FA concentration on the emission of Dox in a PBS buffer (pH = 7.4). The steady-state emission spectra of Dox (5 μg/mL) at neutral pH showed a strong characteristic peak with a maximum at 595 nm upon excitation at 488 nm. The gradual addition of FA to the Dox solution (while keeping Dox concentration constant at 5 μg/mL) led to a continuous decrease in the emission intensity of the Dox (as depicted in Fig. 3) until it reached ~20% (of its initial intensity) at a FA concentration of 203 μM (88.3 μg/mL). These results clearly show that the emissions of Dox are quenched in the presence of FA in solution.

Furthermore, SSL spectra ($\Delta\lambda$ = 30 nm) of Dox (5 μg/mL) at different FA concentrations (Fig. 4), show that not only are the emissions of Dox quenched (at 520 nm) by FA, but also that FA's emissions (at 390 nm) are also quenched by Dox. As shown in Figure 4, the maximum emission of FA shifts from 390 nm at 0.5 μg/mL to 405 nm as the concentration increases to 203 μM (88.3 μg/mL) with a concurrent decrease in its intensity; simultaneously, there is an increase in the emissions at 430 nm as the concentration of FA increases. It is important to note that the increase of FA concentration in the absence of Dox causes only a linear increase in the emission intensity at 390 nm (Fig. 5) without any shift or the emergence of new peaks. These results indicate the presence of strong interactions/binding between FA and Dox leading to the formation of FA-Dox complexes, possibly an exciplex,[18] with an emission at 430 nm in the SSL spectrum. The association constant for the formation of such complexes can be estimated using the Stern-Volmer equation.[14] Since the SSL bands are well defined

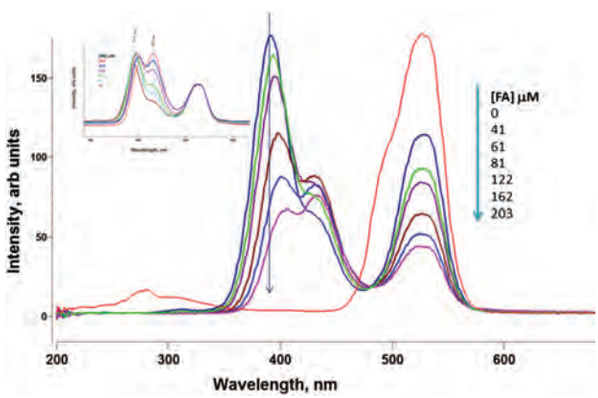


Figure 4. SSL spectra of Dox (5 μg/mL) buffered solution (pH = 7.4) recorded before and after adding various concentrations of FA. Insert is for the normalized spectra at same Dox intensity.

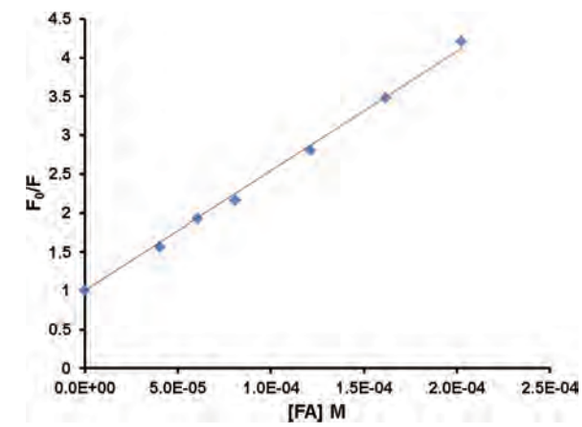
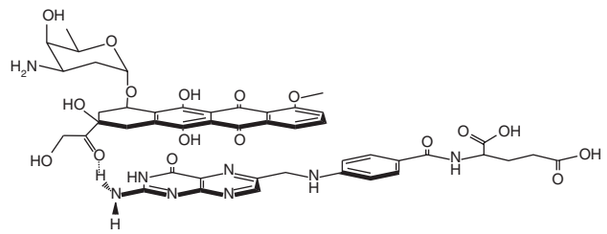


Figure 6. The Stern-Volmer plots of the relative emission intensities of Dox (5 μg/mL) as a function of the FA concentration.



Scheme 2. A Schematic representation of the possible structure of FA-Dox complex formed through π–π stacking and H-bonding.

(see Fig. 4) unlike the steady-state emission bands shown in Figure 3, one can measure the changes in the peak intensity of the defined signals that correspond to Dox emissions at 525 nm. Figure 6 shows a plot of the relative change in the emission intensity of Dox at 525 nm versus the molar concentration of FA; the linear relationship, with a $K_{sv} = 1.5 \times 10^4$ M^{-1}, further supports the presence of a very strong association between Dox and FA.

4. CONCLUSIONS

Folic acid is extensively used in ligand-targeting drug delivery and in many instances with Dox as the chemotherapeutic agent to be delivered. The efficiency of the delivery system is evaluated by monitoring the fluorescence intensity of Dox inside cells using flow cytometry. We investigated the effect of the presence of FA on the fluorescence intensity of Dox in neutral, acidic and basic solutions. The results showed that in all three cases, the fluorescence intensities of both FA and Dox are quenched. Monitoring the emission fluorescence through SSLS at pH = 7.4 and gradually increasing the FA concentration showed a decrease in intensities of both compounds with the appearance of a new peak at 430 nm, while the Stern-Volmer equation produced an association constant of 1.5×10^4 M^{-1}. We thus conclude that, at physiological pH, there is a strong association between FA and Dox leading to the fluorescence quenching of both compounds. Therefore, when Dox and FA are utilized in the same DDS, researchers need to use a correction factor to obtain the correct fluorescence intensity of Dox.

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