Studies on the Interactions Between Genistein and Copolymer F127

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Abstract In this paper, the interactions of an isoflavone molecule, Genistein (Gen), with Pluronic F127 at different pH values have been investigated using laser light scattering techniques, film analysis methods, UV-vis spectroscopy and transmission electron microscopy. The TEM images and the DSL studies indicate the formation of a Gen/F127 complex induced by the solubilization of Gen in micelles, and the stability of the Gen/F127 complex decreases with the increase of pH. At pH of 6.4, the turbidity of the Gen/F127 complex solution is significantly reduced in the presence of 0.31 mol·L⁻¹ ethylene glycol, indicating the existence of hydrogen bonds between Gen and the F127 copolymer. Experiments on controlled release demonstrate that Gen-loaded F127 micelles act as a drug carrier, giving slow release to the surrounding solution over a period of time. Rapid release can be triggered by increasing the pH of the micelle solutions.

Keywords Polymeric micelles · Genistein · Complex · pH dependence · Controlled release

1 Introduction

In recent years, many drug-releasing and drug-targeting systems have been developed to reduce drug degradation, improve the bioavailability and enhance the amount of drug in the area of interest [1, 2]. Among these drug delivery systems, the use of micelles formed by amphiphilic polymers seems to be an interesting alternative to classical formulations [3]. The sizes of polymer micelles, typically between 20 and 100 nm, is effective in avoiding
rapid renal exclusion, but is also small enough to avoid undesirable uptake by the reticuloendothelial system [4]. Therefore, the circulation of the micellar carrier and encapsulated drug is prolonged but since the micelles are composed of polymer chains that are small enough to be eliminated by renal filtration, the eventual disintegration of the micelle will allow the polymer to be excreted. This is important since the long-term build-up of polymer in the body could lead to toxicity. The size and prolonged circulation of polymer micelles also facilitates their passive accumulation at pathological sites such as tumors where the vasculature has increased permeability [5]. Among them, the macromolecular surfactants, Pluronic copolymers, have been studied extensively as drug delivery vehicles due to their excellent biocompatibility [6–9]. Pluronic block copolymers consist of poly (ethylene oxide) (PEO) and poly (propylene oxide) (PPO) blocks arranged in a triblock PEO-PPO-PEO structure. In an aqueous environment, Pluronics form micellar structures with a hydrophobic PPO core, which serves as a microenvironment for the incorporation of lipophilic drugs. The corona shell formed by PEO provides steric stabilization, which enhances the stability and longevity of the drug carriers in blood circulation.

A thorough understanding on the interaction between drug and Pluronic copolymer carriers will be important in optimizing drug delivery, as the controlled drug release correlated directly with interactions of drugs with the polymeric carriers. This forms the basic motivation for the current study. We have selected a Pluronic block copolymer as the platform and an isoflavone drug, Genistein (Gen, Scheme 1) [10] as the model drug for examining the interaction between the drug and the system. In the present study, Pluronic F127 (MW = 12600, EO = 2 × 99 units, PO = 67 units) was chosen as a representative example of the Pluronic family because of its high PEO ratio and narrow polydispersity. PEO provides a steric barrier against self-aggregation and unfavorable interactions with albumin or cellular components in the bloodstream. The drug-loading procedures and drug-polymer interaction were studied using DLS, TEM and UV-vis spectroscopic techniques and film analysis methods. The complex formation of F127 with the drug (Gen) in aqueous solutions was elucidated.

2 Materials and Methods

2.1 Materials

Genistein (Gen, HPLC > 98%) was kindly provided by Nanjing Chemical Reagent Plant (China), and used without further purification. Pluronic® F127 NF (Average formula EO_{99}PO_{67}EO_{99}) was obtained from BASF (Parsippany, NJ). All solutions were prepared from bidistilled Millipore water.

2.2 Prepare Drug-Loaded Micelles

Genistein (5 mg) was added to F127 solutions. The systems were stirred for 12 h at 25°C, and the nonsolubilized fraction was then removed by filtration of the suspension through
a nylon syringe filter (pore diameter 0.45 µm, Teknokroma, Spain). Thus solubilized Gen was assayed by measuring absorbance at 256 nm using a Shimadzu Model UV-2550 spectrophotometer equipped with thermostated quartz cells (1 cm path length) using a validated method. Gen calibration curves were developed by dissolving a given amount of Gen in methanol followed by the required dilution by water. The presence of methanol did not alter either the extinction coefficient or the specific wavelength at which the maximum in UV absorbance appeared. The calibration curves yielded the following absorbance-concentration dependencies:

\[
C_{\text{gen}} = \frac{A_{256} - 0.0203}{0.201} \times 10^{-5}
\]

(1)

2.3 Transmission Electron Microscopy (TEM)

TEM observation was carried out (TECNAI-12 TEM, Philips, the Netherlands) in the polymer solution, which was negatively stained with 2% phosphotungstic acid and placed on a copper grid coated with film.

2.4 Dynamic Light Scattering (DLS)

Measurements were carried out at a scattering angle of 90° using an ALV 5022 laser light-scattering (LLS) instrument equipped with a cylindrical He-Ne laser (model 1145p-3083; output power = 22 mW at \( \lambda = 632.8 \) nm) in combination with an ALV SP-86 digital correlator with a sampling time range of 25 ns–40 ms. The LLS cell was held in a thermostated index-matching vat, filled with purified dust-free toluene, with the temperature controlled within ±0.02 °C. All solutions were filtered through a Millipore filter with a 0.45 mm pore size and thermostated at 25 °C for at least 0.5 h. Experiment duration was 10 min and each experiment was repeated two or more times. The time correlation function of the scattered intensity \( G_2(t) = I(t)I(t+\Delta t) \) was analyzed with the CONTIN program to calculate the distribution function of the decay times. The apparent hydrodynamic radius, \( R_{h\text{app}} \), was determined using the Stokes-Einstein equation,

\[
R_{h\text{app}} = \frac{\kappa T}{6\pi \eta D}
\]

(2)

where \( D \) is the translational diffusion coefficient, which is calculated from the decay rate \( \Gamma = Dq^2 \), \( k \) is the Boltzmann constant, \( \eta \) is the solvent viscosity, \( q \) is the scattering vector \( (q = 4\pi n \sin(\theta/2)/\lambda) \), \( n \) is the refractive index of the solution, \( \theta \) is the scattering angle, and \( \lambda \) is the wavelength in a vacuum of the incident laser light.

2.5 Structural Characterization of Gen-Polymer Systems Through Film Analysis

Films obtained by drying of filtered 3% (w/v) polymer micellar solutions containing solubilized Gen (see section on the preparation of the drug-loaded micelles) at 40 °C for 24 h, were characterized by differential scanning calorimeter (DSC), FTIR spectroscopy and X-ray diffraction. The DSC experiments were carried out in triplicate using a DSC Q100 (Netzsch DSC-204 F1) with a refrigerated cooling accessory and modulated capability. Samples (5–6 mg) were analyzed in aluminum-closed pans. The calorimeter was calibrated for baseline. Samples were heated from 25 to 300 °C at 5 °C/min. Infrared spectra were recorded over a range of 400–4000 cm\(^{-1}\), using a Bruker IFS 66V FTIR spectrometer (Germany). Dried samples were mixed with KBr and pressed into tablets. X-ray diffraction data were recorded on a Bruker D8 Advance X-Ray Polycrystalline Diffractometer at a rate of 1.5°2θ/min, in the range between 5 and 60° 2θ, using Cu Kα radiation.
2.6 In Vitro Release

The in vitro release study was conducted in the way reported by Xiong et al. [11], briefly: 3 mL of Gen/F127 or Gen aqueous solution were put in the dialysis membrane (cut-off 12,000–14,000 Da). The sealed dialysis membrane placed aqueous in buffer solutions (100 mL). The whole solution was then placed in a shaking water bath at 37 °C for the drug release study. At predetermined time intervals samples (3 mL) of the solution outside the dialysis membrane were taken out and their absorbance measured at 257 nm, using a UV-vis scanning spectrophotometer, to determine the concentration of Gen. Fresh solution (3 mL) was added to replenish the sample that was removed in order to maintain a constant volume.

3 Results and Discussions

3.1 Gen-F127 Complex Formation

Gen is chosen as the model drug. It is a known isoflavone molecule and is reported to have tumor enhancing effects on breast and colon cancer [12, 13]. At 25 °C, the solubility of Gen in water at pH = 7 is about $3 \times 10^{-6}$ mol·L$^{-1}$. The hydroxyl groups in the Gen molecule could undergo deprotonation at higher pH, which would increase the solubility of Gen but the solubility of Gen is still low at pH = 10 (about $1.8 \times 10^{-4}$ mol·L$^{-1}$, determined from our experimental data by the UV-vis spectra method). Figure 1 illustrates the solubilization of Gen into F127 micelles. When Gen is dissolved in water at pH = 7, it forms a transparent solution (see Fig. 1a). On addition of F127 solution, the copolymer-drug solution becomes turbid and bluish as is shown in Fig. 1b. F127 micelles provide hydrophobic micro-domains, which solubilize small organic molecules, such as Genistein. Along with the solubility study, the bluish turbid solution of Gen-loaded micelles is strong evidence for the Gen/F127 complex formation.

The dynamic light scattering and transmission electron microscopic experiments were also carried out to prove the formation of Gen/F127 complex. The drug contents of micelle solutions are measured by UV spectroscopy. DLS characterization of the drug-loaded micelles after passing though 0.45 µm filter indicated reasonably narrow size distributions (Fig. 2). Before the addition of Gen, the hydrodynamic diameter of empty micelles is about...
Fig. 2 Particle size distribution of the Gen/F127 complex from dynamic light scattering. 
(a) Empty micelles, wt-%
F\textsubscript{127} = 3%. (b) Gen-loaded F127 micelle, [Gen] = 0.08 mmol·L\textsuperscript{−1}, wt-%
F\textsubscript{127} = 3%

Fig. 3 TEM image of Gen/F127 complex. (a) Empty micelles, wt-%
F\textsubscript{127} = 3%, scale bar = 50 nm. (b) Gen-loaded F127 micelle, [Gen] = 0.08 mmol·L\textsuperscript{−1}, wt-%
F\textsubscript{127} = 3%, scale bar = 0.5 µm. Sample directly prepared from DLS experiments

8 nm. When Gen is added, the diameter of the drug-loaded micelles increases rapidly and is found to be in the range of 200–400 nm. The increase in micelle size due to the solubilized drug has been predicted by a recent mathematical simulation of drug solubilization [14]. Figure 3 shows the TEM micrographs depicting the morphology of F127 micelles in the presence and absence of Gen. As shown in Fig. 3a, spherical F127 micelles are produced at pH = 7. After the addition of drug molecules, large and compact complexes are observed (Fig. 3b) indicating that the formation of Gen/F127 complexes induced by Gen solubiliza-
tion. The diameter of the complexes is about 200 nm, which is consistent with that determined from dynamic light scattering.

3.2 Structural Characterization of Gen-Polymer Systems Through Film Analysis

To gain mechanistic insight into the possible interactions of Gen with the F127, films obtained by drying of F127 dispersions containing solubilized Gen are analyzed by DSC, X-ray diffraction and FTIR spectroscopy. The thermal behavior of the films can provide information about the drug–polymer compatibility, miscibility and any alteration in the chemical structure of either polymer or drug [15, 16]. If the drug remains in the solid state in the polymer surfactant aqueous solution, the melting point \( T_m \) of the drug and the glass transition \( T_g \) of the polymer would remain unchanged. In contrast, if the drug is molecularly dissolved in the polymer surfactant solution, the \( T_g \) of the polymer is typically lowered and the drug melting transition is absent [15]. The thermogram of Gen shows a melting peak at 280 °C (Fig. 4). This peak is not observed in the thermograms of the films, indicating a complete solubilization of the drug inside of the micelles, with the consequent loss of crystallinity. This is confirmed by the absence of characteristic peaks pertaining to Gen in the X-ray diffractograms of the film (Fig. 5). The thermograms of F127 films show a typical melting peak of Pluronic at 50 °C. This endothermic peak shifts to a lower temperature in the presence of Gen, which is a clear indication of the polymer/drug miscibility and complex formation. It has previously been reported that the interaction of small hydrophobic drugs to non-ionic surfactants such as Tween and Pluronic occurred through hydrogen bonding and hydrophobic interactions [17]. In our system, Gen has aromatic rings, and its solubility in water is low; therefore, Gen will be easily extracted into the F127 micelles through hydrophobic interaction. However, Gen is known to have a varying degree of hydroxylation, and it has been shown that the hydroxyl groups have a high capacity to form hydrogen...
bonds [18], and the F127 molecule is composed of PEO and PPO blocks. So we conclude that besides hydrophobic interactions, the hydrogen bonding between hydroxyl groups of Gen and the ethoxylated PEO chains also promotes the aggregation process in the Gen/F127 complex.

Many studies have used FTIR to probe interaction and/or compatibility in polymer–polymer or polymer–drug blends [19]. In our studies, the polymer/Gen complex film was prepared to assess the extent of polymer–drug interaction at the molecular level. The FTIR spectra of both Gen and the polymers alone are shown in Fig. 6. The peaks characteristic of functional groups likely to engage in hydrogen bonding interaction are labeled. In the FTIR spectra of the physical mixtures of polymer and drug there are no obvious shifts in any peaks that correspond to functional groups capable of hydrogen bonding (data not shown). However, in the spectra for the polymer–drug films there are slight shifts in peaks characteristic of the polymer. The presence of shifts or changes in the spectra for the polymer–drug films is an indication that interactions exist between the polymer and drug; for example, the infrared band in 1112 cm$^{-1}$ is assigned to C-O-C vibration, and which is shifted to a lower vibration frequency (1103 cm$^{-1}$) in the film. The shift of this bond proves that the hydrogen bonding interactions are present in the polymer–drug film [20]. So, in agreement with our discussions above, there are interactions between Gen and F127 driven by H-bond and hydrophobic interactions.

3.3 The pH Dependence of Gen/F127 Complex Formation

Because a hydrogen-bonding interaction is strongly affected by the solution’s pH, we studied the effect of solution pH on the Gen/F127 complex formation. Figure 7 shows the transmittance measurement of polymer/drug complex, as $\% T$, plotted against pH values; it can be seen that the transmittance intensity at 600 nm changes with the pH of the solution. The $\% T$ of the polymer/drug complex is found to be almost unchanged in the range $0 < \text{pH} < 7$, and then decreases sharply at $\text{pH} > 7.0$, consistent with the dissociation of Gen/F127 aggregates, above $\text{pH} = 12$, then the $\% T$ remains nearly unchanged. However, the Gen/F127 complex is completely reversible in the sense that cycling the pH back to the acidic will rehomogenize the drug/polymer solution and the bluish turbidity rapidly returns. On the basis of the
results obtained from turbidity measurements, the observed aggregation of Gen/F127 is a pH-dependent process. Genistein molecules process three free hydroxyl groups with $pK_a$ values of 7.2, 10.0 and 13.1 respectively. At lower pH values, Gen molecules are not ionized.
The scheme of the complexation of Gen/F127 with the change of pH and the complex is stabilized by the H-bonding interaction between hydroxyl groups of Gen and the ethylene oxide groups in the PEO chains. Upon pH increase, H-bonding interaction decreases due to increasing number of ionized Gen molecules. The reduced H-bonding leads to the dissociation of the Gen/F127 aggregates observed at higher pH value. This result indicates that the H-bonding interactions play a major role in drug loading in the F127 system. The possible interaction mechanism is schematically illustrated in Scheme 2. However, one might venture to ask why hydrophobic interaction does not effect the complex formation between Gen and micelle (i.e., with increasing pH, the mole fraction of dissociated forms of Gen increases, which results both in the solubility of Gen in the continuous water phase increasing and the dissociation of the Gen/F127 aggregates). We measured the distribution coefficients ($K_D$, $K_D$ is the distribution coefficient of Gen between the F127 micellar phase and the continuous water phase) at different solution pH values. The method used for calculating the partition coefficients was described in our recent papers (see Eqs. 5 and 6 of [21] and Eq. 1 of [22]). The results are shown in Fig. 8, where the distribution constant $K_D$ of Gen between micelle and the continuous water phase decreased with increasing pH. The estimated $K_D$ value at lower pH is higher than that at higher pH probably because of the ionization of Gen, which behaves as a tribasic acid with $pK_a$ of 7.2, 10.0 and 13.1 for the $C_7$, $C_4'$ and $C_5$ atoms, respectively [10]. From the data of Fig. 8, it can be expected that Gen should be totally incorporated into F127 micelles at pH = 3.8 ($K_D = 2193.8$), and about
99% of the total added Gen is incorporated into micelles when pH = 12.5 ($K_D = 221.9$). So, even at the higher pH values, almost all Gen molecules would be incorporated into F127 micelles and hydrophobic interactions still exists in the system at higher pH values.

To confirm the existence of H-bonding interactions between F127 and Gen, the UV transmittance of the F127/Gen (pH = 6.4) complex in the presence and absence of ethylene glycol is examined. In the absence of ethylene glycol, the transmittance of the F127 complex is 54.4%, and it increases to 78.8% in the presence of 0.31 mol·L$^{-1}$ ethylene glycol. The ethylene glycol is a strong H-bonding agent, which can disrupt H-bonding by forming H-bonds with both proton donors and acceptors [23]. The increase in the transmittance intensity of the complex, with the addition of ethylene glycol, suggests that inter- or intramolecular H-bonded complexes are present between Gen and F127 copolymer at pH = 6.4, which is consistent with the results obtained by film analysis.

During storage of the drug-loaded micelle solutions for five days, Gen is gradually released as a precipitate and the transmittance intensity is increased from an initial value of 54.4% to around 69.5%. The initial average hydrodynamic radius of the Gen/F127 complex is 273 nm, and decreases to 199 nm five days later. This controlled release of the drug from the micelle cores shows that the entrapped Gen is thermodynamically unstable relative to the precipitated Gen. Essentiality, the drug-loaded micelles are kinetically stable and thermodynamically unstable [24]. Drug release is driven by the change in chemical potential of the Gen. Meanwhile, the size of the drug-micelles decreases, as expected, because of the drug loss from the cores.

### 3.4 pH-Dependent Release of the Drug from the Complex

Figure 9 shows typical results of drug release from micelles. Because of the low solubility of Gen at the pH of the micellar solutions, a blank release experiment (curve a in Fig. 9) for Gen without micelles was performed at pH = 10 using an equivalent amount of drug ($1.5 \times 10^{-4}$ mol·L$^{-1}$) to that trapped in the micelle solution. It is assumed that the Gen
diffusion rate in the buffer solution does not change with the solution pH. This experiment measures the permeation of the Gen through the dialysis membrane in the absence of any control by the micelles. From Fig. 9, the comparison between the blank and micellized drug release demonstrates that drug release from micelles in a pH = 4 buffer (curve c) is slower than the drug alone dissolved in a pH = 10 buffer (curve a). After 1 h, the cumulative curves show that about 42.1% of Gen is released in the absence of micelles, which is about three times the Gen released from the micelles. This retardation effect on the rate of Gen diffusion demonstrates the controlled release properties of the F127 micelles.

It has been shown that formation of the Gen/F127 complex depended on the pH of the solutions. Due to the greatly increased solubility of Gen at high pH, the pH-triggered Gen/F127 complex dissociation suggests that fast, triggered release of Gen should be feasible. To investigate this possibility, a Gen-loaded micelle solution is prepared at pH = 9 and is then placed in pH = 9 buffer. As shown in Fig. 9, its release (curve b) is only slightly slower than the blank drug release (curve a) but is faster than the drug release rate at pH = 4 (curve c). The slightly retarded release rate is related to the high stability of the Gen/F127 complex in acid media where hydrogen bonding in the complex is maintained. The Pluronic block copolymer used for the drug-polymer formation in this work are widely used as a biocompatible materials with low toxicity, and this type of pH-controlled complexation could provide a new platform methodology in the area of drug delivery.

4 Conclusions

The pH-induced complex formation between F127 micelle and Genistein was investigated in an aqueous phase. The complexation began at lower pH values through hydrogen-bonding to produce turbid bluish micelle solutions and pH increase resulted in the dissociation of the Gen/F127 complex. Furthermore, DSC, XRD and FTIR spectroscopy of films confirmed the
interactions between Gen and copolymer. This pH-dependent complexation was a reversible process and storage of drug-loaded micelle solutions led to the slow release of the Gen, over a period of days, driven by precipitation of the soluble component of the drug in equilibrium with the micelles. Drug release profiles obtained by monitoring diffusion through a membrane, using UV spectroscopy, indicated that the Gen in the F127 micelles could be slowly released into a surrounding solution with no drug in it. Rapid, triggered release results when the solution pH was switched to 9, to dissociate the complex. Thus, these micelle systems offered potential for both controlled and pH-triggered carrier.

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References
