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Effects of Triton X-100 on Properties of Hemoglobin and Controlled Release of Drug in Hemoglobin/Ribavirin/H₂O System

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Abstract: Effects of Triton X-100 on the properties of hemoglobin (Hb) and on the controlled release of ribavirin were studied using the methods of UV-Vis spectrum, fluorescence spectrum, zeta potential, conductivity, high-performance liquid chromatographic (HPLC), and image morphology in Hb/ribavirin/H₂O system. With the increase of concentration of Triton X-100 in the system, the intrinsic fluorescence intensity, synchronous fluorescence intensity, fluorescence polarization, zeta potential, and morphology of Hb all changed gradually, and the ribavirin located on the Hb surface was dissociated and released out. When the concentration of Triton X-100 was higher than 1×10⁻⁵ mol·L⁻¹, the stronger interaction of Triton X-100 with Hb was predominant. Hb was unfolded and denaturized. A little Triton X-100 can protect Hb from the effects of ribavirin.

Key Words: Hemoglobin; Property; Ribavirin; Controlled release; Triton X-100

Drugs can interact and associate with native proteins and cells, and can also affect the behaviors of proteins and cells. So, when drug interacts with and kills viruses, it also has a similar effect on the native proteins and cells in the human body. The interaction and the association of drug with protein restrict and affect the drug metabolism and distribution as well as drug efficacy. The site of the interaction and association is mainly at the residues of the alkaline amino acid[1,2]. Generally, the interaction is mostly electrostatic action, hydrogen bonding, hydrophobic action, and van der Waal force[3,4]. If the interaction and association can be changed or modulated so as to decrease some side-effects of drugs on man, they can be very important and beneficial for man.

Surfactants and their orderly aggregations can provide more applications in various fields, such as daily chemical engineering, food, material science, and life science[5,6]. They always act with the native cells and proteins in the human body to some extent[7⁻¹⁸]. Some scientists studied and mimicked some life systems through the formation of micelles, microemulsions, vesicles, and lipid crystals by surfactants[19⁻²²]. We have recently reported the interaction of methylene blue (bioactive molecule) with BSA in the SDS micelle system and the influence of low cetyltrimethylammonium bromide concentration on the interactions and properties of hemoglobin with acyclovir[23,24].

Hemoglobin (Hb) is one of the important proteins in the human body, main ingredient in the erythrocyte. It is a tetrameric protein with a molecular mass of 64500 Da, consisting of two identical α-chains of 141 amino acids each and two identical β-chains of 146 amino acids each[25,26]. Its main physiological function is to transport oxygen to tissue in the body. The isoelectric point of Hb is approximately 6.5. Ribavirin (R) is a depressor for the dehydrogenation enzyme of hypoxanthine nucleotide phosphate. It has antiviral functions for some diseases such as aspiratory disease, flu, simplicity bleb viral disease, hepatitis C. Ribavirin also can halt replication of the West Nile virus in cell culture[27] and cure SARS (severe acute respiratory syndrome)[28,29]. However, there are some side-effects when ribavirin is used to treat diseases, such as stomach discomfort, anemia, and the decrease of hemoglo-
bin level\[5,30,31\].

In the present article, the influences of Triton X-100 (T, nonionic surfactant) on the behaviors of Hb in Hb/ribavirin/H$_2$O system were studied to obtain the effects of nonionic surfactant on the association of Hb with drug and the controlled release of surfactant to drug in order to regulate efficiently the interaction between ribavirin and Hb and the concentration of the free drug, and to reduce some side-effects of drug on human information for the improvement of the metabolism and distribution of drug in life science, biotechnology, medicine, molecular functional design, and new drug development.

1 Materials and Methods

1.1 Materials

Triton X-100 was purchased from Aldrich (>99%, Milwaukee, WI). Hb was purchased from Shanghai Lizhu Dongfeng Biotechnology Co. Ltd., China (B.R.), ribavirin from Hubei Qianjiang Pharmaceutical Co. Ltd., China (99%, material drug). Doubly distilled and deionized water was used for the preparation of the solutions.

1.2 UV-Vis spectrum determination

UV-Vis spectra and difference spectra of Hb with ribavirin were determined using UV-2501PC UV-Vis spectrum spectrometer (Shimadzu Co., Japan). The range of scanning wavelength was 700–190 nm.

1.3 High-performance liquid chromatographic (HPLC) determination

The contents of the compositions in Hb/ribavirin/Triton X-100/H$_2$O system were measured using a CLASS-VP HPLC system (Shimadzu Co., Japan) with model SPD-M10AVP UV-Vis detectors and an autosampler. The chromatographic data were analyzed using CLASS-VP chromatographic manager (Shimadzu). A Shim-pack CLC-ODS column (150 mm × 6 mm × 10 µm) was used for the HPLC separations. The flow rate was set to 1.0 mL/min$^{-1}$ and the effluent was monitored using UV-Vis double-wavelength detection. The mobile phase was 90% methanol and 10% water.

1.4 Fluorescence spectrum determination

Both the fluorescence emission spectrum and the synchronous fluorescence spectrum of Hb were determined at the excitation wavelength (280 nm) using RF-5301 fluorescence spectrometer (Shimadzu Co., Japan). The wavelength differences (Δλ) between the excitation and the emission wavelengths of Hb were 15 nm and 60 nm in the synchronous fluorescence.

1.5 Conductivity determination

The conductivity of the system was measured by DDS-11A conductometer (Shanghai No.2 Analytical Instrument Co., China). The conductometer was calibrated using 0.01 mol·L$^{-1}$ KCl solution.

1.6 Zeta potential of Hb determination

The protein electrophoresis may be carried out under the condition of electric field. The zeta potential of Hb was measured from the electrophoresis of Hb by Js-94F micro-electrophoresis-meter (Shanghai Zhongcheng Instrument Co., China) connected with a P4 computer. The principle method of the zeta potential measurement was similar to that given in reference[17]. The measured voltage was 10–30 V, stabilizing time 5 min, and measuring interval 50 s. The measured difference was within ±0.2 mV.

1.7 Negative-staining technology

Living organisms are always composed of lightly staining elements, and hence their image contrast by TEM observation is low. Negative-staining technology can overcome the low image contrast. The samples with protein were prepared by directly dipping copper mesh grids previously coated with a poly(vinyl formal) (or Formvar) film into the appropriate dispersions[52]. After the sample was adsorbed on the Formvar film for approximately 15–20 min, dripping imaging solution (such as phosphor tungstenic acid, PTA) on the Formvar film with protein Hb. The staining time was about 1–2 min. The copper mesh grids were then dried. The size and morphology of the protein can be clearly observed using TECNAIR Transmission Electron Microscopy (Philip Apparatus Co., USA).

All of the above measurements were carried out at (25.0±0.1) °C. The pH value of the measured samples was 7.0.

2 Results and discussion

2.1 Effect of ribavirin on Hb behaviors

There is an absorption peak at approximately 276 nm for most proteins, which mainly corresponds to tryptophan and tyrosine. The peaks of metHb are at 406 and 630 nm, hemichrome at 500 nm, hemachrome monomer at 630 nm, and oxyHb at 576 nm[15,33]. With the increase of ribavirin concentration, both the peak absorbencies at 630 and 406 nm increase slightly, whereas the absorbency at 500 nm decreases slightly (Fig.1). An isosbestic point is reached at approximately 550 nm. So, ribavirin affects on Hb structure, i.e., the content of metHb increases but the content of hemichrome decreases slightly. There does not appear new absorption peak, which implies that ribavirin and Hb may not form a complex. The absorption peak of ribavirin appears only at 208 nm. The absorbency of ribavirin in the Hb/ribavirin/H$_2$O system is 6%–8% less than that in the same concentration of ribavirin in the ribavirin/H$_2$O system. From the HPLC determination, the
percentage of free ribavirin is found to be 62%–65% in the Hb/ribavirin/H2O system. Therefore, 6%–8% ribavirin may be embedded in the interior of Hb and approximately 26% is strongly adsorbed on the Hb surface.

With the increase of ribavirin concentration (cR), the intrinsic fluorescence intensity of Hb (at 333.6 nm) decreases rapidly at first and then slowly (Fig.2). When cR reaches approximately 2.5×10⁻⁵ mol·L⁻¹, the intensity changes little. On one hand, there is a furan ribose group and a ternary-nitrogen imidazole in ribavirin molecule. So, the interaction of ribavirin with Hb involves hydrophilic action and hydrogen bonding. Ribavirin changes the Hb micro-environment and the residues of the amino acids move into the inner of Hb. These lead to the decrease in fluorescence intensity. On the other hand, ribavirin may be adsorbed or located on Hb surface, which causes the residues of Hb to be partially shielded. These also lead to the decrease of fluorescence intensity. At high ribavirin concentration, the interaction and the shield gradually reach a saturation point and equilibrium. So, the fluorescence intensity decreases slowly with the increase of ribavirin concentration.

In 5×10⁻⁶ mol·L⁻¹ Hb and 1×10⁻⁵ mol·L⁻¹ ribavirin system, from the relationship of the fluorescence intensity of Hb with time, it is found that the half life of the fluorescence quenching of ribavirin to Hb is 4.85 min. After approximately 35 min, the intensity changes very little. An interaction equilibrium is experimentally reached between ribavirin and Hb.

The synchronous fluorescence spectra of protein at Δλ=15 nm and Δλ=60 nm can indicate the micro-environment of the tryptophan and the tyrosine in the protein, respectively[34–36]. Fig. 3 shows that the synchronous fluorescence intensities of Hb at Δλ=15 nm and Δλ=60 nm vary with ribavirin concentration. From Fig.3, with the increase of ribavirin concentration, the two intensities decrease. These results indicate that tryptophan and tyrosine may be gradually moved into the Hb core or may be shielded by ribavirin.

For a dynamic quenching, generally, the higher the temperature, the greater is the molecular vibrancy energy. The more the molecular collision probability, the more is the molecular activity, and the less the system viscosity, the less is the fluorescence efficiency and the less is the fluorescence intensity[34–36]. When the system temperature changes from 25 to 37 °C, the intrinsic fluorescence intensity and the synchronous fluorescence intensity of Hb all decrease in the Hb/ribavirin/H2O system. From these, it is obvious that the quenching process of ribavirin to Hb is dynamic.

The quenching process of ribavirin to Hb can be treated using Stern-Volmer equation[34–36],

\[
\frac{F_0}{F} = 1 + Kc_Q
\]

where \(F\) and \(F_0\) are the fluorescence intensities of the substance with and without quencher, respectively, and \(c_Q\) is the concentration of quencher. For dynamic quenching, \(K\) is called Stern-Volmer constant, corresponding to the ratio of bimolecular quenching constant to monomer-molecule dissociation.
rate constant. The $K$ of the dynamic quenching for ribavirin to Hb, calculated from the plot of $F_0/F$ versus $c_R$, is approximately $1510 \text{ mol}^{-1}\cdot\text{L}$ at low ribavirin concentration. The $K$ value is not high. So, the interaction of Hb with ribavirin is not very strong.

With the increase of ribavirin concentration, the zeta potential of Hb increases but the net negative-charge of Hb decreases (Fig.4(a)). The apparent net charge and the zeta potential change from negative to positive charge. The conductivity of the Hb/ribavirin/H$_2$O system first decreases and then increases (Fig.4(b)). In the ribavirin molecule, the furan ribose group displays weak acidity, whereas the ternary-nitrogen imidazole shows weak alkalinity. The probability that the ternary-nitrogen imidazole gets a proton is slightly more than the probability that the furan ribose group loses a proton. Therefore, ribavirin displays weak positive charge in aqueous solution. When ribavirin interacts with Hb, the charge of the Hb surface must decrease. Whereas ribavirin can adsorb onto the Hb surface through electrostatic action and hydrogen bonding. The Hb surface charge is partially shielded by ribavirin. So, the net Hb surface charge decreases as well. When ribavirin concentration increases, the zeta potential increases. If the concentration of ribavirin is high, the interaction and the shielded state gradually reach saturation and equilbrium. Therefore, the apparent zeta potential increases slightly.

Here, we must point out that Hb can experimentally exist for some time, but cannot aggregate and separate out from the aqueous solution even if the zeta potential appears to be zero. Hb is still surrounded by hydrophilic medium because of the stronger hydrophilic action and hydrogen bonding between Hb and ribavirin. This phenomenon is also observed and demonstrated by the electrophoresis of Hb.

### 2.2 Effect of Triton X-100 on Hb properties

There are many ethoxy groups in Triton X-100 molecule. Because of the hydrophilic interaction, hydrogen bonding, and van der Waals’ action between Hb and Triton X-100, Triton X-100 increases both the UV-Vis peak at 276 nm and the intrinsic fluorescence of Hb. The micro-environments of the tryptophan and tyrosine in Hb are also altered. The system viscosity increases and the heat vibrancy of Hb decreases. There does not appear new peak in the UV-Vis spectrum. The effects of Triton X-100 on the zeta potential of Hb and the system conductivity are very obvious (Fig.5). With the increase of Triton X-100 concentration, the charge of Hb decreases and the system conductivity increases accordingly. When Triton X-100 concentration is more than $5\times10^{-6} \text{ mol}\cdot\text{L}^{-1}$, the fluorescence intensity increases and the peak shifts to blue. If the polarity of the solution decreases, the peak of the tryptophan shifts to blue and the fluorescence intensity increases. The tryptophan must be adsorbed onto the protein surface or must reach protein surface as a result of solvent abdution$^{[35]}$, that is, Triton X-100 results in the exposure of the tryptophan in Hb.

#### 2.3 Effects of Triton X-100 on Hb properties in the Hb/ribavirin/H$_2$O system

With the increase of Triton X-100 concentration in the Hb/ribavirin/H$_2$O system, the UV-Vis absorbances of Hb at 630 and 406 nm decrease slightly, whereas that at 500 nm increases slightly (Fig.6). These indicate that Triton X-100 can modulate the Hb structure from metHb to hemichrome and lead to the partial recovery of the original structure of Hb.
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In the Hb/ribavirin/H2O system, the intrinsic fluorescence intensity and the synchronous fluorescence intensities of Hb at Δλ=15 nm and Δλ=60 nm increase gradually with the increase of Triton X-100 concentration (Fig.7). These results imply that the residues of the tryptophan and tyrosine in Hb are gradually moved from the inner to the surface of Hb. When Triton X-100 concentration increases to (2−5)×10⁻⁶ mol·L⁻¹, all the intensities can be almost returned to that while Hb solely exists in the same concentration of Hb. When Triton X-100 concentration is higher than 1×10⁻⁵ mol·L⁻¹, the intensities increase. The fluorescence peaks shift to blue. Here, the relationship between the intensities and Triton X-100 concentration is almost consistent with that in the Hb/Triton X-100/H2O system. Because the interaction of Triton X-100 with Hb is stronger than that of ribavirin with Hb, Triton X-100 may gradually replace the ribavirin located on the Hb surface, thereby resulting in the release of the ribavirin. The amino acids packed in the interior of Hb by ribavirin are gently exposed and partially returned to their original sites. The conformation of Hb is partially rebounded. When Triton X-100 concentration is quite high, the stronger interaction of Triton X-100 with Hb shows an obvious predominance.

The influence of Triton X-100 on the structure and the micro-environment of Hb can be further proved by the fluorescence polarization of Hb, $P$, (2)

$$P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp},$$

where $I_\parallel$ and $I_\perp$ are the fluorescence intensities of the polarized components while they are mutually parallel and perpendicular between the polarizer and polarimeter, respectively. In the Hb/ribavirin/H2O system, the polarization increases first slowly and then rapidly with Triton X-100 concentration (Fig. 8). The decrease in interaction between ribavirin and Hb makes the freedom of movement of Hb and the polarization increases. On one hand, when Triton X-100 is added to the system, ribavirin located on the Hb surface is released out. On the other hand, the viscosity of the system increases with the increase of Triton X-100 concentration. The decrease of the freedom of movement of Hb also results in the increase in polarization. Therefore, the polarization apparently changes slightly if Triton X-100 is less than 5×10⁻⁶ mol·L⁻¹. When the concentration of Triton X-100 is larger than 1×10⁻⁵ mol·L⁻¹, the effect of Triton X-100 is predominant, and the polarization shows an obvious change.

Fig. 9 shows the effects of Triton X-100 on the zeta potential of Hb and the system conductivity in the Hb/ribavirin/Triton X-100/H2O system. With the increase of Triton X-100 concentration, the zeta potential decreases rapidly at first and then slowly. The Triton X-100 can decrease the dielectric constant of the system, thereby resulting in the release of ribavirin. The tryptophan and tyrosine wrapped in the interior of Hb by ribavirin are drawn back to the original states by the stronger polarization. Therefore, the polarization apparently changes slightly if Triton X-100 is less than 5×10⁻⁶ mol·L⁻¹. When the concentration of Triton X-100 is larger than 1×10⁻⁵ mol·L⁻¹, the effect of Triton X-100 is predominant, and the polarization shows an obvious change.

**Fig.6 UV-Vis different spectra of Hb vary with Triton X-100 concentration**

Triton X-100 concentrations (mol·L⁻¹): (a) 0, (b) 1×10⁻⁷, (c) 1×10⁻⁸, (d) 5×10⁻⁶, (e) 1×10⁻⁵, (f) 5×10⁻⁵, and (g) 1×10⁻⁴. Concentrations of Hb and ribavirin are 5×10⁻⁵ and 1×10⁻⁹ mol·L⁻¹, respectively.

**Fig.7 Synchronous fluorescence intensities of Hb vary with Triton X-100 concentration**

Filled and open squares correspond to fluorescence intensities at Δλ=60 nm and λ=343 nm. Filled and open circles correspond to fluorescence intensities at Δλ=15 nm and λ=307 nm. Filled squares and circles correspond to fluorescence intensities at 25 °C. Open squares and circles correspond to fluorescence intensities at 37 °C. Concentrations of Hb and ribavirin are 5×10⁻⁵ and 1×10⁻³ mol·L⁻¹, respectively.

**Fig.8 Fluorescence polarization of Hb varies with Triton X-100 concentration**

Filled squares and circles correspond to Hb concentrations of 1×10⁻⁶ and 5×10⁻⁵ mol·L⁻¹, respectively.
interaction of Triton X-100 with Hb. Therefore, the negative charge and zeta potential of Hb decrease and the conductivity increases. The Hb structure may gradually return to the original state. When Triton X-100 concentration increases to the range of \((2-5) \times 10^{-6}\) mol\(\cdot\)L\(^{-1}\), the zeta potential rebounds to its original value. If the Triton X-100 concentration is very high, the stronger interaction of Triton X-100 with Hb is the most dominant. The zeta potential shows a continuous decrease.

The Hb morphology influenced and modulated by Triton X-100 can be directly observed from the negative-staining TEM (Fig.10). From Fig.10, it can be seen that ribavirin and Triton X-100 all interact with Hb and change the Hb morphology. The original spherical morphology of Hb is destroyed and a loose structure is formed. The peptide of Hb shows a wide distribution. With the increase of Triton X-100 concentration, Triton X-100 can lead to the recovery of the loose structure because of the release of ribavirin. The Hb morphology gradually tends to return to its original spherical morphology. However, when Triton X-100 concentration is high, the effect of Triton X-100 on the morphology is dominant. The morphology again shows an unfolded or loose structure. These results are all in accordance with the above results.

2.4 Effects of Triton X-100 on controlled release of ribavirin in Hb/ribavirin/H\(_2\)O system

The interaction of Hb with Triton X-100 results in the dissociation and release of the ribavirin adsorbed on the Hb surface. From the HPLC of the Hb/ribavirin/Triton X-100/H\(_2\)O system, the percentage of free ribavirin increases from 62% to 89% with the increase of the Triton X-100 concentration. Fig.11 shows that the Hb concentration depends on the Triton X-100 concentration, \(c_T(r_c)\), required to recover completely the Hb behavior in the system. \(c_T(r_c)\) increases linearly with Hb concentration. The slope of the straight line is approximately 1.5, from which it is obvious that the binding of Hb with a particular number of Triton X-100 results in the recovery of the behaviors of Hb. In addition, the linear relationship indicated by the line that passed through the origin point indicates that most of the Triton X-100 added is bound to the protein. The mixing ratios at low Triton X-100 concentration can be regarded as the actual binding number of the surfactant, i.e., 1.5. The Triton X-100 molecules are bound to an Hb molecule. The binding is not steady because no new peak is found in the
UV-Vis and fluorescence spectra of Hb. As is well known, the binding behaviors of a surfactant to a protein were extensively examined in the initial stage. The present statement is slightly contradictory to the results reported so far: even over a low surfactant-concentration range, the binding to a protein has generally been accepted to proceed according to an equilibrium relationship with a free surfactant monomer of a particular concentration. Here, it should be noted that the above estimation is made using the changes in behavior of the protein induced by the binding of the surfactant.

The HPLC of the Hb/ribavirin/Triton X-100/H2O system shows that the percentage of free ribavirin, \( f_r \), linearly increases with the increase of Triton-X-100 concentration (Fig.12). This indicates that Triton X-100 almost interacts with Hb and results in the release of the ribavirin adsorbed on the Hb surface. The binding site of Triton X-100 with Hb is similar or nearly similar to the binding site of ribavirin with Hb. When ribavirin concentration increases to approximately 82% of the total ribavirin concentration, \( f_r \) shows a slow and nonlinear increase. Some molecules of ribavirin embedded in Hb are still released out because more Triton X-100 induces changes in the Hb structure. From Fig.12, it can be seen that \( f_r \) is still less than 95% when Triton-X-100 concentration is much higher than the ribavirin concentration. Approximately 5% ribavirin may enter into the cavity of Hb, which is not quickly replaced by Triton X-100 having high density and long chain length.

The turning concentration \( c_T(t) \) of Triton-X-100 in Fig.12 also linearly increases with ribavirin concentration (Fig.13). This illustrates that all the molecules of Triton-X-100 interacts with Hb to replace and release the ribavirin bound to Hb. When the ribavirin concentration is higher than 1.5 \( \times 10^{-5} \) mol\( \cdot L^{-1} \), the curve shows a deflection from the straight line. The higher the ribavirin concentration, the higher is the content of the ribavirin embedded in Hb and the higher is the content of Triton X-100 applied to release the ribavirin.

3 Conclusions

Triton X-100 can effectively regulate the interaction between Hb and ribavirin and the release of the ribavirin located on the Hb surface so as to control the metabolism and distribution of the drug and the drug efficacy. Ribavirin facilitates the transformation of Hb from hemichrome to metHb. Whereas, Triton X-100 transforms metHb into hemichrome.

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