Research Article

Interaction of Mitoxantrone-Loaded Cholesterol Modified Pullulan Nanoparticles with Human Serum Albumin and Effect on Drug Release

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To clarify nanoparticle-protein interaction and their action characteristics, the interactions between MTO-CHP NPs and human serum albumin (HSA) were studied by isothermal titration calorimetry (ITC), fluorescence spectroscopy, dynamic light scattering (DLS), and circular dichroism spectroscopy (CD). Hydrophobically modified pullulan (CHP) nanoparticles (NPs) loaded with mitoxantrone (MTO) were prepared (MTO-CHP NPs) with size 166.9 nm. The spherical shape was verified by transmission electron microscopy (TEM). The ITC results demonstrated an interaction between MTO-CHP NPs mainly by hydrophobic interaction force, electrostatic force, and hydrogen bonding. The mean binding constant $K_A$ was $8.32 \times 10^4$ M$^{-1}$ and mean HSA coverage $0.939 \pm 0.302$. MTO-CHP NPs could quench the fluorescence intensity of HSA, which gradually decreased to be balanced in 9 h and indicated the completion of the complexation. The size and zeta potential changes of the combined particle were dynamically detected with DLS at 0, 3, 6, 9, 12, 15, and 18 h. When the reaction was completed at 9 h, the particle size and potential remained stable, accompanied by a size change from 89.91 to about 145 nm and potential change from -15 to -3 mV, respectively. The results of CD measurement showed that the change in ellipticity of HSA at 208 nm was similar to the fluorescence spectra and DLS measurements with MTO-CHP NPs combined with HSA. At the beginning of the reaction, the proportion of $\alpha$-helix was 52.3% to 43.7%, which decreased by 39.1% at compound stabilization. The release of MTO from MTO-CHP NPs at pH = 5.6 was significantly accelerated, whereas that of MTO from HSA-MTO-CHP NPs was significantly reduced, and the drug release was significantly slowed down even under acidic conditions, which indicates the beneficial effect of HSA on the persistence and stability of the HSA-MTO-CHP NP compound.

1. Introduction

Nanopreparations have good sustained release and targeting and are a hot topic of research in drug delivery systems [1, 2]. The nanometer size can be used to passively target tumor tissue via an enhanced permeability and retention effect, achieve local fixed-point release, and exert an antitumor effect [3]. Nanopreparations enter the blood circulation by intravenous injection and are susceptible to adsorption of plasma proteins before passively targeting tumor tissues [4]. The adsorption of nanoparticles (NPs) by plasma proteins changes the characteristics of the NPs, such as size, surface charge, and surface hydrophobicity, which alters their biological behaviors in vivo and thus their efficacy [5, 6]. The type and quantity of plasma protein adsorption are closely related to the nature of the NPs themselves. The surface of NPs mainly adsorbs high-abundance proteins and proteins with strong adsorption to NPs [7, 8].

Human serum albumin (HSA) contains more than 60% high-abundance proteins in serum. HSA has three domains I, II, and III—each with two subdomains, A and B [9]. It can bind to various endogenous and exogenous compounds
via amino acid residues and mainly binds to hydrophobic cavities at IIa (site I) and IIa (site II) [10]. HSA binding is an important factor in helping to transport drugs and limiting their free active concentrations [11, 12]. Many studies have investigated the reaction of small molecule drugs and macromolecular drugs with HSA. Small-molecule drugs such as palmatine react with HSA, which will affect the tissue distribution, metabolism, and effect of palmatine [9]. Macromolecular substances such as fluorescent carbon dots (CDs) of novel carbon-based nanomaterials react with HSA, affecting the actual medical application of CDs and weakening the biological activity of HSA [13].

Recently, some HSA NPs as a new drug delivery systems show potential clinical value, such as targeting tumor or inflamed tissues with high stability, high biodegradability, low toxicity, and immunogenicity [14]. Zhang et al. used amphiphilic poly(N-(2-hydroxypropyl)-methacrylamide) copolymer NPs (pHPMA NPs) loaded with doxorubicin (DOX) or pHPPMA NPs without DOX in the HSA solution under a physiological environment [15]. Unlike unloaded NPs, drug-loaded NPs did not interact with HSA, so the delivery of DOX by using pHPPMA NPs in the human body is not affected by the presence of HSA [15]. However, the interaction of HSA with drug-loaded NPs may affect the targeting and sustained drug release properties. For example, morin (3,5,7,2′,4′-pentahydroxyflavone)-loaded poly(lactic-co-glycolic acid) (PLGA) NPs (MPNPs) combined with HSA could promote morin’s continuous release [16]. Therefore, the study of the interaction between drug-loaded NPs and HSA is of great significance to the research and optimization of drug-loaded nanopreparations.

In this study, we used cholesteric modified pullulan (CHP) NPs loaded with mitoxantrone (MTO) as drug-loaded NPs to explore the interaction with HSA. MTO is an anthracycline antibiotic that has been used as an antitumor drug in clinical practice. It has no selectivity for tumor and HSA could promote morin’s continuous release [16]. Therefore, the study of the interaction between drug-loaded NPs and HSA is of great significance to the research and optimization of drug-loaded nanopreparations.

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temperature 25°C and constant pressure to atmosphere. At the beginning of the titration, the syringe was placed in the sample cell and dripped at regular intervals to ensure balanced sample action. The thermodynamic parameters were then obtained and displayed in the curve. The raw data are expressed as thermal changes over time. Three sets of experiments were performed: the first group with 0.45 mM MTO dropped into 0.002 mM HSA, the second group with 0.15 mM HSA dropped into 0.01 mM CHP, and the third group with 0.9 mM HSA dropped into 0.01 mM CHP-MTO. Before the curve was fitted, the first data point was always deleted from the data set, and the data were analyzed by using the Origin 8.5 software. A set of site models was used to obtain the composite stability constant \( (K_A) \) and molar reaction metamorphosis \( (\Delta H^\circ) \). Then, the standard free energy \( (\Delta G^\circ) \) and entropy change \( (\Delta S^\circ) \) were calculated as:

\[
\Delta G^\circ = -RT\ln K_A = \Delta H^\circ - T\Delta S^\circ.
\]

The enthalpy change and entropy change reflect the interaction mode and mechanism [26].

(2) Fluorescence Spectroscopy. HSA contains a tryptophan that is excited in the ultraviolet region to emit fluorescence, forming a fluorescent spectrum. When other molecules interact with HSA, tryptophan fluorescence may change, depending on the effect of this interaction on protein conformation [27, 28]. HSA and MTO, CHP NPs, and MTO-CHP NPs were mixed and reacted at a molecular ratio of 3.6:1 (HSA: MTO). The obtained mixture was placed in a 2 mL Eppendorf tube (EP tube) and shaken at 20 rpm, 25°C, for 24 h for full reaction. Fluorescence spectrophotometry (Shimadzu RF-4500, Japan) was used to record the fluorescence intensity of free HSA and HSA that began to bind or was already bound to MTO, CHP NPs, and MTO-CHP NPs. The maximum changes in fluorescence intensity with recombination time were recorded. The tryptophan chromophore in the HSA molecule was excited at 290-450 nm. The excitation and emission slit widths were 5 and 12 nm, respectively.

(3) Circular Dichroism (CD). In biological fluids, the secondary structure of human serum albumin may be toxic because of changes in drug-induced conformation and function. Because CD spectroscopy is sensitive to changes in protein structure [29], it was used to study the structural changes of HSA after the compound formation.

The CHP-HSA complex was prepared by storing the complex in a 2 mL EP tube, keeping it at a constant temperature of 25°C and stirring on a shaking table at 12 rpm for 18 h. The CD spectra (at wavelength 200-250 nm) of free HSA, HSA after the addition of MTO, CHP NPs, and MTO-CHP NPs were recorded by using a CD spectrometer (Jasco J-810, Japan) at 37°C with a 0.1 cm cuvette cell. \( \alpha \)-Helix \( (\alpha-H) \) was calculated as follows [30]:

\[
\alpha-H \text{ (\%)} = \frac{MRE_{208} - 4000}{33000 - 4000} \times 100,
\]

where \( MRE_{208} \) is the average residual ellipticity at 208 nm (deg cm$^2$ dmol$^{-1}$), \( n \) is the number of HSA amino acid residues (585), \( C_p \) is the molar concentration of HSA (1.5 x 10$^{-5}$ mol/L), and \( L \) is the length of the cuvette cell (0.1 cm).

2.2.4. Determining Drug Release. The standard curve of MTO was obtained by using a microplate spectrophotometer (UV-384 Plus, Molecular Devices, USA). The NPs were dialyzed in phosphate-buffered saline (pH = 7.4) and acidic solution (pH = 5.6) simulating the tumor microenvironment to study MTO release in vitro. In brief, the solution of CHP-NPs loaded with MTO and HSA-MTO-CHP-NPs (2 mg/mL) was placed in a VISLING dialysis tube (molecular weight cut-off 12-14 kDa, USA) and dialyzed in an air-bath shaker for 50 rpm at 37°C. At a predetermined time, 5 mL released medium was removed with a pipette and 5 mL fresh medium was added. MTO release was determined at 608 nm by UV spectrophotometry followed by calculating the cumulative release percentage (Q%) [31].

3. Results

3.1. FT-IR Analysis. As shown in Figure 1, CHP showed a distinct ester carbonyl absorption peak near 1735 cm$^{-1}$, while the carboxyl peak near 1708 cm$^{-1}$ disappeared in the infrared spectrum of CHS. The absorption peaks of methyl and methylene groups near 2930 cm$^{-1}$ were significantly enhanced, indicating that CHS was grafted on to pullulan.

3.2. Characterization of Drug-Loaded CHP NPs. The average size distribution and polydispersity index (PDI) of MTO-CHP NPs was 166.9 nm and 0.213, respectively (Figure 2(b)), so the NPs were stable and the particle size was uniform. As compared with CHP NPs (-1.21 mV) [7],...
the mean zeta potential of MTO-CHP NPs was −0.698 ± 6.09 (mV) and higher (Figure 2(a)), so MTO decreased the electronegativity of CHP NPs, which corresponded to the positive electrochemistry of MTO due to the presence of imino groups [32]. The TEM image shows the spherical shape of MTO-CHP NPs (Figure 2(c)).

3.3. ITC-Determined Characterizations of Free Drugs, CHP NPs, and Drug-Loaded CHP NPs Interacting with HSA.

Figure 3(a) shows MTO titration into HSA. The lines present positive and negative peaks during the titration process. At each titration, the positive peaks were almost the same, about 0.1125 μcal/sec, whereas the absolute value of negative peaks decreased with increasing drops from the second drop. The corresponding curve showed an increasing trend, and the dots were scattered and undulating.

Corresponding thermodynamic parameters could be obtained from the curve. According to \( \Delta G = \Delta H^r - T\Delta S^r \), \( \Delta G < 0 \) indicates that the reaction proceeded spontaneously for MTO binding HSA via hydrophobic force, electrostatic interaction, and hydrogen, and \( \Delta H^r < 0 \) and \( \Delta S^r > 0 \) indicate that the adsorption of MTO and HSA was mainly due to electrostatic interaction [33, 34]. MTO is electropositive due to its imino group, whereas HSA is electronegative. The mean coverage \( N \) of MTO on HSA was 12.4 ± 1.02, and the mean binding constant \( K_A \) was \( (77.9 \pm 5.37) \times 10^5 \text{M}^{-1} \) (Table 1), so MTO had high affinity with HSA. With the MTO dropped, the reactions released or absorbed heat, but the whole reaction was an endothermic process (Figure 3(a)).

This finding may be due to the molar concentration of HSA being much less than that of MTO, which is diluted when the MTO is dropped, and the dilution process releases heat for hydration. The amount of heat release during each titration did not change, so the adsorption combination was independent of the dilution exotherm, and the reaction was mainly endothermic.

Figure 3(b) shows the HSA titration into CHP NPs. During the titration process, the alternate lines show the positive and negative peaks, and the total area of the positive peaks was definitely larger than the total area of the negative peaks. The value of the negative peaks was basically unchanged at each titration, maintained at about -0.0125 μcal/sec; the value of the positive peak decreased with increasing drops. The curve shows a downward trend, and the dots were scattered but steady and close to the curve. \( \Delta H^r > 0 \) and \( \Delta S^r > 0 \) indicate that the interaction between HSA and CHP NPs was mainly caused by hydrophobic interactions [33, 35]. CHP contains an inner hydrophobic core and an outer shell of hydrophilic groups, whereas HSA has hydrophobic amino acid residues. Therefore, CHP and HSA are close and bind with each other because of the hydrophobic interaction. According to \( \Delta G = \Delta H^r - T\Delta S^r \), \( \Delta G < 0 \) indicates that the adsorption reaction of HSA and CHP NPs proceeded spontaneously by the hydrophobic interaction. The mean coverage of HSA on CHP NPs was 1.17 ± 0.044, and the mean \( K_A \) was \( (27.7 \pm 3.26) \times 10^4 \text{M}^{-1} \) (Table 1), so the affinity between the two was high. The curve had an endothermic and exothermic part, but the latter was definitely larger than
the former, finally showing an exothermic appearance. The endothermic part is likely due to the weak electronegativity of CHP NPs, which exerts the repulsive force of the same charge with HSA helping the system absorb minute amounts of heat during the HSA titration each time.

Figure 3(c) shows the HSA titrating into MTO-CHP NPs. The graph shows two small negative peaks at the first two drops in the experiment, which did not appear after that. A positive peak appeared after the first drop, and its value decreased with increasing drops. The corresponding curve

**Figure 3**: Isothermal calorimetry data for HSA titration into (a) MTO, (b) CHP, and (c) MTO-CHP NPs at 25°C.
had a downward trend, and the dots were neat, basically falling on the curve. On the basis of \( \Delta H^\circ > 0, \Delta S^\circ > 0 \), to \( \Delta G^\circ < 0 \), the reaction proceeded spontaneously, and the main driving force in the reaction was hydrophobic interaction between the two. The mean coverage of HSA on drug-loaded NPs was 0.939 \( \pm \) 0.302, and the mean \( K_A \) was \( (8.32 \pm 2.78) \times 10^4 \) M\(^{-1} \) (Table 1), so the affinity between the two was low. The curve does not show an endothermic phenomenon except for the first two drops, showing an exothermic phenomenon, because HSA takes the surface amino acid residues as active sites, adsorbs on the surface of MTO-CHP NPs by the hydrophobic reaction, and then gradually complexes into its hydrogel matrix. A part of the heat release should be considered to result from the emptying of the system was increased, and the free energy in the corresponding unit contact area was reduced. The endothermic part in the curve is likely due to the combination between the HSA molecule and the free MTO released from the MTO-CHP. Because the free MTO is only a small portion and the HSA increase goes faster than the drug release while the HSA is continuously titrated, gradually, the exothermic combinations of HSA and CHP NPs are predominant.

Both the coverage and binding constants were greater in Figure 3(a) than in Figure 3(b), so the affinity of HSA for MTO was higher than that of HSA for CHP NPs likely because of the electropotitivity of MTO, electronegativity of HSA, and weak electronegativity of CHP NPs. HSA is easier to combine with oppositely charged species, so the affinity with MTO was higher.

Figures 3(a) and 3(c) show that the two binding effects were endothermic and exothermic, respectively. The binding constant and coverage were much higher in Figure 3(a) than in Figure 3(c), so the affinity of HSA was much higher for MTO than for MTO-CHP NPs. This finding may be due to MTO being encapsulated in CHP NPs, and the affinity of CHP NPs being much lower for HSA than MTO, resulting in decreased affinity for MTO-CHP NPs.

In contrast, Figures 3(b) and 3(c) both show exothermic phenomena. However, the binding constants and coverage were definitely less in Figure 3(c) than in Figure 3(b), reflecting the lower affinity of HSA for MTO-CHP NPs than CHP. This finding may be due to the competition between MTO and HSA in combination with CHP. MTO occupies the binding site of HSA on CHP NPs, and the binding of the two is restricted by each other, for decreased affinity of HSA for MTO-CHP NPs.

3.4. Fluorescence Spectroscopy Determination of Interactions of Drug, CHP, and Drug-Loaded CHP NPs with HSA. The maximum value of fluorescence emission intensities was at 342 nm owing to the effect of the tryptophan residue in the HSA molecule [27], so CHP NPs and HSA formed a complex. When the NPs were completely combined with HSA and at the beginning of the process, the fluorescence intensity for HSA was greatly decreased, and MTO-CHP NPs more strongly quenched HSA fluorescence intensity than did CHP NPs.

The maximum fluorescence intensity of HSA varied with complexation time (Figure 4(c)). When MTO was combined with HSA, the fluorescence intensity decreased within 3 h, then remained stable, so the complexation of MTO and HSA completed quickly. When HSA was combined with CHP or MTO-CHP, the fluorescence intensity decreased rapidly within 0-6 h and slowly within 6-18 h. The initially rapid decrease in fluorescence intensity was due to the fast adsorption of HSA to NPs, and the slow decrease was due to the gradual complexation between them. Therefore, we considered the compound obtained at 15 h as a completely formed complex.

3.5. Characterization of the Compound of HSA with Drug-Loaded CHP NPs. The mean size distribution, PDI, and zeta potential changes for MTO-CHP NPs with HSA at 0, 3, 6, 9, 12, 15, and 18 h are in Figures 5(a) and 5(b) and Table 2. From 0 to 12 h, the mean particle size increased from 89.91 to 144.4 nm, which indicates the interaction between HSA and MTO-CHP NPs and the integration of HSA. From 12 to 18 h, the increase in mean particle size slowed, so HSA was basically complexed with MTO-CHP NPs. PDI changed from 0.508 and 0.514 at the beginning of the reaction to 0.210 after 15 h of reaction, because multiple HSA was rapidly adsorbed on the surface of MTO-CHP NPs and there were many substances in the composite system. When HSA...
adsorbed on the surface gradually merged into the core of the NPs, the composite reaction was completed, and the dispersion was gradually stabilized with the decreased PDI value.

The zeta potential gradually changed from -15 to -0.307 mV, because the charge of HSA was -15 mV [36], whereas MTO-CHP NPs were almost uncharged, which further indicated a composite reaction between HSA and MTO-CHP NPs. The charge was redistributed by electrostatic interaction, and the absolute value of the overall potential decreased.

TEM revealed that MTO-CHP NPs remained spherical after 15 h of reaction with HSA (Figure 5(c)). HSA did not change the morphology of MTO-CHP NPs. HSA may enter into the core of MTO-CHP NPs or be evenly distributed on the surface of MTO-CHP NPs.

3.6. Changes in Secondary Structure with Drug-Loaded CHP NPs Combined with HSA. The CD spectra for HSA shows a negative peak at 208 nm, considered as a characteristic peak of the α-helical structure [37]. The proportion of α-helix contents for free HSA was 52.3%, and that at the beginning of the adsorption of MTO, CHP, and MTO-CHP decreased to 50.0%, 46.9%, and 43.7%, respectively (Table 3). The proportion after maximum adsorption of MTO, CHP, and MTO-CHP was 49.4%, 41.8%, and 39.1%, respectively (Figures 6(a) and 6(b)). The ellipticity at 208 nm was almost unchanged after 3 h when HSA interacted with MTO but was stabilized until 9 h when HSA interacted with CHP and MTO-CHP (Figure 6(c)). Thus, the HSA adsorption of MTO was rapid; moreover, MTO changed the secondary structure of HSA to a small extent. The combination of HSA with CHP and MTO-CHP was slow, and HSA complexed into its hydrogel matrix gradually. During this process, HSA was pulled by the hydrophobic interaction of the CHP hydrophobic core and the hydrophilic polysaccharide chain of the CHP surface, causing the HSA to deform and altering its secondary structure. Because of the double complex reaction between MTO-CHP and HSA (i.e., the interaction between CHP and HSA, MTO, and HSA), the secondary structure of HSA changed to a greater extent.

Figure 4: Fluorescence spectra for HSA in phosphate buffer with pH 7.40 at 25°C. (a) a → d represents the fluorescence spectra of the beginning of reaction for HSA, HSA and MTO, HSA and CHP NPs, and HSA and MTO-CHP NPs, respectively. (b) a → c represents the fluorescence spectra for HSA and MTO, HSA and CHP NPs, and HSA and MTO-CHP NPs, respectively, after the reaction was completed at 15 h. (c) Change in maximum fluorescence intensity as a function of time combined with MTO (a), CHP NPs (b), and MTO-CHP NPs (c).
3.7. Sustained Drug Release after HSA Complexed with Drug-Loaded CHP NPs. Figure 7 shows the MTO release profile of MTO-CHP NPs in media with pH 5.6 or 7.4 and a composite with HSA in media with pH 5.6 or 7.4. MTO release had a biphasic character; that is, the drug was released rapidly in the first 12 h, at 56.73%, 39.82%, 32.45%, and 28.02%, respectively, and the drug release rate was slowed down within 12 to 48 h, with cumulative release percentages of 68.75%, 50.54%, 35.22%, and 31.68%, respectively. Because NPs capture hydrophobic drugs in two forms of loading, surface adsorption, and core loading, the surface-adsorbed drug was rapidly released, whereas the core-loaded drug was slowly and continuously released [38]. The drug release of MTO-CHP NPs was much slower. This finding might have two explanations. First, HSA is rapidly bound to the surface of CHP NPs and then slowly enters the NP core and binds to the CHP hydrophobic domain, forming steric hindrance and weakening the hydrophobic interaction between MTO and CHP. Second, during the MTO release process, it may combine with the HSA adsorbed by the NPs. The drug release of MTO-CHP NPs was faster at pH 5.6 than at pH 7.4, mainly because the degree of protonation of MTO and water solubility became larger under...
However, the MTO-CHP NPs complexed with HSA had a low drug release rate under both pH 7.4 (physiological condition) and pH 5.6 (acidic condition) because the steric hindrance of HSA formation is large, and this property is not affected by acidity or alkalinity.

4. Discussion

HSA can react with small molecular drugs, NPs, and drug-loaded NPs [40–42]. However, a number of fundamental principles have not been elucidated, including the binding force, binding coefficient, type of reaction, relationship between active and passive adsorption, and characteristics of their interactions [43–45]. The combination of HSA with drugs, NPs, and drug-loaded NPs definitely affects their structure, distribution, toxicity, and effectiveness of related drugs [46]. Understanding the characteristics of HSA-associated interactions, especially the HSA effect on drug release from drug-loaded NPs, holds great potential for improving drug delivery in the clinical applications.

We prepared CHP NPs loaded with MTO (MTO-CHP NPs) with size 166.9 nm. The interaction between acidic conditions [39]. However, the MTO-CHP NPs complexed with HSA had a low drug release rate under both pH 7.4 (physiological condition) and pH 5.6 (acidic condition) because the steric hindrance of HSA formation is large, and this property is not affected by acidity or alkalinity.

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MTO-CHP NPs was mainly by hydrophobic interaction force, electrostatic force, and hydrogen bond. MTO-CHP NPs could quench the fluorescence intensity of HSA. And the fluorescence intensity gradually decreased to be balanced at 9 h. At the same time, the particle size and potential remained stable, accompanied by a size change from 89.91 to about 145 nm and potential change from -15 to -3 mV. With MTO-CHP NPs combined with HSA, the change in ellipticity of HSA at 208 nm was similar to that of fluorescence spectra and DLS measurements. The proportion of α-helixes was decreased until combination for 9 h. The release of MTO from MTO-CHP NPs at pH = 5.6 was significantly accelerated, whereas that of MTO from HSA-MTO-CHP NPs was significantly reduced. And the drug release was significantly slowed down even under acidic conditions, which indicates the beneficial effect of HSA on the persistence and stability of the HSA-MTO-CHP NP compound.

In general, the interaction of HSA with small molecular drugs, such as naproxen, ibuprofen, and flurbiprofen, is relatively simple, rapid, and reversible, reaching a saturation state in a short time [47]. Two mechanisms are believed involved in the interaction between NPs and HSA. The first is the "nanocrystalline corona," whereby small NPs produce a corona on the surface of the protein [48]. The second is the "protein corona," whereby proteins form a corona around NPs. The combination of different NPs cannot be separated from electrostatic interaction, van der Waals force, hydrogen bond, and hydrophobic interaction [49].

The reaction between drug-loaded NPs and HSA is complicated. Some drugs form strong space resistance and block the reaction of HSA with drug-loaded NPs. For example, after pHMPA NPs are loaded with DOX, the structure cannot be captured by the network structure formed by the pHMPA chain or diffused through the loose pHMPA NP shell into the cholesterol core of pHMPA NPs [15]. Ghosh et al. studied the interaction of MPNPs and HSA with morin. Morin in the MPNP surface initially bound to HSA, forming morin-HSA, which in turn bound to the NP surface. Morin encapsulated in the MPNP matrix may also be adsorbed by the complexed HSA, resulting in a surface-induced steric resistance effect, which limited the release of morin from MPNPs [16].

In the present study, we systematically investigated the reaction of MTO-CHP NPs with HSA. As shown in Figure 2, HSA interacted with MTO-CHP NPs similar to CHP NPs and HSA. Because of the hydrophobic interaction, HSA was rapidly adsorbed to the surface of MTO-CHP NPs by electrostatic interaction. Because of the hydrophobic section of the NP core and the resistance of the hydrophilic polysaccharide chain on its surface, HSA gradually complexed into the core of NPs and combined with the cholesterol group, forming HSA-MTO-CHP NPs. In DOX-loaded pHMPA NPs, the hydrophobicity of DOX is smaller than that of MTO, and DOX is distributed in the pHMPA shell, which results in large steric resistance hindering the adsorption of HSA [15]. However, the mean coverage of HSA and MTO-CHP NPs was 0.939 ± 0.302, and the mean binding constant was 0.832 ± 0.278 × 10^4 M⁻¹, which was much smaller than that for HSA and CHP NPs. The fluorescence quenching of HSA was evident, accompanied by a significant decrease in α-helix content in HSA.

According to previous research [22], MTO can react with HSA and stabilize domain I in the hydrophobic region of HSA via hydrophobic interaction, hydrogen bonding, and electrostatic interaction. The CHP NPs package MTO prepared by dialysis self-assembly, physical adsorption, or MTO-coated CHP NPs, and the latter is encapsulated in the hydrophobic core of CHP NPs via hydrophobic interaction. So, the interaction between HSA and MTO-CHP NPs may differ from that of HSA and CHP NPs for two reasons. First, MTO reacts with HSA. When the combined drug is released, drug release is hindered by the presence of HSA. In addition, the high affinity between MTO and HSA indicates that MTO is secondarily combined with HSA adsorbed on CHP NPs. This result is consistent with Ghosh et al. [16]. Second, HSA competes with MTO. Both HSA and MTO bind to the hydrophobic region of CHP NPs via hydrophobic interaction. Our ITC results showed that the affinity and coverage of HSA and MTO-CHP NPs were relatively low. All features met our expectations.

Blood contains opsonins, integrins, immunoglobulins, complement proteins, fibrinogens, etc., which are easily adsorbed on the surface of NPs, thereby promoting the recognition and phagocytosis of NPs by macrophages and removing NPs from the blood or their accumulation in immune organs such as liver, spleen, and bone marrow. The targeting of drug-loaded nanometers is interfered by combination with these proteins [50, 51]. However, HSA-complexed NPs could block the absorption of other serum proteins including opsonin and integrin [52], thereby prolonging the blood circulation of drug-loaded NPs in vivo and enhancing their passive targeting.

The design and application of nanoformulations require understanding the interaction between drug-loaded NPs and HSA. HSA-complexed NPs can be used as an ideal model to explore the potential applications of albumin nanosystems in vivo.

5. Conclusion

This study found that MTO-CHP NPs interact with HSA, with hydrophobic interactions playing a major role. Combining HSA and MTO-CHP NPs is a slow process. Because of the hydrophobic interaction, HAS first adsorbs on the surface of CHP-MTO NPs, then gradually complexes into its hydrogel matrix, resulting in the increased size and potential changes of MTO-CHP NPs. In addition, the combination alters the secondary structure of HSA and its α-helix content. In addition, the complexation of HSA enhances the sustained MTO release from MTO-CHP NPs, and the drug release adapts to acidic conditions, thereby improving the stability of the drug release.

Abbreviations

- CHP: Hydrophobically modified pullulan
- MTO: Mitoxantrone
- NPs: Nanoparticles
HSA: Human serum albumin  
MTO-CHP NPs: Mitoxantrone-loaded hydrophobically modified pullulan NPs  
HSA-MTO-CHP NPs: HSA combined with MTO-CHP NPs.

Data Availability
The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Authors’ Contributions
Liming Yuan and Bu Guo contributed equally to this work.

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