Supplementary Material

Anticancer drug delivery systems based on specific interactions between albumin and polyglycerol

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Experimental details

General.

All solvents and reagents were purchased from commercial sources (Merck, Sigma Aldrich, and Fluka) and were used directly without further purification. β -CD-g-PG was synthesized according to our previous report.¹ IR spectra were measured with a AgBr cell in the range of 400–4000 cm⁻¹ by means of a Jasco FT-IR/410 spectrofotometer (Jasco, USA). UV-visible measurements were performed using a Shimadzu 1650 PC scanning spectrophotometer (Shimadzu, Japan). Circular dichroism (CD) spectra were recorded with a Aviv-215 spectrometer (Aviv, USA). Dynamic light scattering and zeta potential measurements were performed with a NanoBrook 90Plus Zeta Particle Size Analyzer Zeta (Brookhaven, USA). Fluorescence quenching were performed using a Cary Eclipse Fluorescence Spectrophotometer (Varian, Australia). ¹³C, ¹H NMR spectra were recorded on a 400 MHz Bruker spectrometer (Bruker, USA) with TMS as internal standard.

Sample preparation.

All solutions were prepared in 0.1 M PBS. In a typical procedure, stock solutions of HSA were prepared in PBS at pH 7.4. Then, stock solutions of β -CD-g-PG (0.1 g in 1 ml PBS) were prepared. Solutions of β -CD-g-PG at various concentrations were added to the HSA solution. In all experiments, the total concentration of HSA in each vial was kept constant to 2×10^{-6} M. The vials containing the samples were subsequently placed on a shaker, slowly rotated for 60 min, and then allowed to stabilized for further 60 min. The reference solution was also prepared according to procedure described above yet in the absence of β -CD-g-PG. A series of HSA/ β -CD-PG/DOX and of HSA/ β -CD-PG/PTX solutions were prepared by mixing β -CD-g-PG/HSA solutions with solutions containing either drug at different concentrations. Each resulting mixture was shaken for the time required to ensure the formation of a homogeneous solution and, then, dialyzed against PBS of pH 7.4 for 1 h.

UV-visible spectra.

The Shimadzu UV-2450 spectrophotometer (Shimadzu, Japan) equipped with a 1.0 cm quartz cell was used for recording the UV-vis spectra in the wavelength range 200 to 800 nm. Aliquots of 100μ L of the β -CD-g-PG solution at different concentration were employed for each measurement. All spectra were recorded at 37°C.

Fluorescence quenching measurements.

Intrinsic HSA fluorescence quenching induced by β -CD-g-PG was recorded on a Cary Eclipse fluorescence spectrophotometer (Varian Australia). Excitation was performed at 280 nm (95% excitation Trp-214), and the emission wavelength was recorded from 300 to 450 nm. The slit width was 2.5 nm. Fluorescence emission was measured at 37 °C in PBS containing various concentrations of β -CD-g-PG (from 0.26 to 2.37×10⁻³ M). The concentration of HSA was fixed at 2×10^{-6} M.

Circular Dichroism.

Circular dichroism (CD) spectra were recorded in PBS solution at 37 °C on a Aviv-215 spectropolarimeter (Aviv, USA), using a quartz cell and in the 200-280 nm range. The β -CD-g-PG solution was added in small aliquots to protein solution. HSA concentration was kept constant (2 ×10⁻⁶ M) while varying β -CD-g-PG concentration (0.26×10⁻³ to 2.1×10⁻³ M). The α -helix, β -sheet, β -turn, and random coil contents of each sample were calculated from the corresponding CD spectra and using CDNN 2.1 software.

Fourier Transformed IR spectroscopy (FT-IR).

FT-IR spectra were recorded on a Jasco FT-IR/410 spectrophotometer (Jasco, USA). Solution of β -CD-g-PG (0.06 g in 0.6 ml PBS) was added dropwise to the HSA solution (2 ×10⁻⁶ M PBS at pH =7.4) with constant shaking to ensure the formation of homogeneous solution. Spectra were collected after 1h incubation of HSA with β -CD-g-PG solution at 37°C. The same method was used for the preparation of the corresponding β -CD-g-PG/HSA/drug complexes (DOX and PTX).

HSA/β -CD-g-PG binding constant from UV-vis spectra.

The value of the binding constants K_b between β -CD-g-PG and HSA was also estimated using the available UV-vis data following the method reported in Zhong et al. and Stephanos.² By assuming the establishment of only one type of interaction between β -CD-g-PG and HSA in solution solution, the supramolecular association can be expressed as:

$$HSA + \beta - CD - g - PG \leftrightarrow HSA - \beta - CD - g - PG$$
(1)

for which the binding constant K_b reads:

$$K_b = \frac{[\text{HSA}-\beta-\text{CD}-\text{g}-\text{PG}]}{[\text{HSA}][\beta-\text{CD}-\text{g}-\text{PG}]}$$
(2)

Setting [HSA– β -CD-g-PG] = C_B , Eq. (2) becomes:

$$K_{b} = \frac{c_{B}}{(c_{HSA} - c_{B})(c_{\beta - CD - g - PG} - c_{B})}$$
(3)

where C_{HSA} and $C_{\beta-CD-g-PG}$ are the analytical concentrations of HSA and β -CD-g-PG in solution, respectively. According to Lambert-Beer's law:

$$C_{HSA} = \frac{A_0}{\varepsilon_{HSA} \times L} \tag{4}$$

$$C_B = \frac{A - A_0}{\varepsilon_B \times L} \tag{5}$$

in which A_0 and A are the absorbance of HSA at 280 nm in the absence and presence of β -CD-g-PG, respectively, ε_{HSA} and ε_B are the molar extinction coefficients of HSA and the HSA- β -CD-g-PG complex, and *L* is the light path of the cuvette (1 cm).

Substituting Eqs. (4) and (5) in Eq. (3), after a simple rearrangement Eq. (6) can be formulated as:

$$\frac{A_0}{A-A_0} = \frac{\varepsilon_{HSA}}{\varepsilon_B} + \frac{\varepsilon_{HSA}}{\varepsilon_B \times K_b} \cdot \frac{1}{C_{HSA}}$$
(6)

According to Eq. (6), the plot of $1/(A-A_0)$ vs. $1/C_{\beta-CD-gPG}$ gives a straight line, from which the binding constant K_b can be estimated as the ratio of the intercept and the slope.

Doxorubicin and paclitaxel loading by HSA/β-CD-g-PG.

DOX (1mg, 1.8×10^{-6} mol) or PTX (1mg, 1.1×10^{-6} mol) were added to a solution of HSA/ β -CD-g-PG (0.06 g β -CD-g-PG, dissolved in 600 μ L of PBS + 5 mL of HSA 2 μ M). Thereafter, the reaction mixtures were purified by dialysis (1000 MWCO) against water for 1h at 37°C in the darkness to remove unbound DOX and PTX. Freshly prepared solutions of the β -CD-g-PG/HSA/DOX and β -

CD-g-PG/HSA/PTX were used to determine the amount of loaded drugs using UV-vis absorbance at λ_{max} = 490 nm and λ_{max} = 227 nm, respectively.

Atomistic Molecular Dynamics Simulation of the HSA/β-CD-g-PG Molecules and their complexes with doxorubicin and paclitaxel.

All atomistic simulations and data analysis were performed with the AMBER 14 suite of programs.³ The models of the β -CD-g-PG, DOX, and PTX compounds were built and geometryoptimized using the Antechamber module of AMBER 14 and the GAFF force field.⁴ The molecule structures were solvated in a TIP3P⁵ water box. Then, the required amount of Na⁺ and Cl⁻ ions were added to neutralize the system and to mimic salt conditions, removing eventual overlapping water molecules. The solvated systems were subjected to a combination of steepest descent/conjugate gradient minimization of the potential energy, during which all bad contacts were relieved. The relaxed systems were then gradually heated to 298 K in three intervals by running constant volume-constant temperature (NVT) MD simulation, allowing a 0.5 ns interval per 100 K. Subsequently, 10 ns MD simulations under isobaric-isothermal (NPT) conditions were conducted to fully equilibrate each solvated compound. The SHAKE algorithm⁶ with a geometric tolerance of 5×10^{-4} Å was imposed on all covalent bonds involving hydrogen atoms. Temperature control was achieved using the Langevin⁷ temperature equilibration scheme and an integration time step of 2 fs. At this point, these MD runs were followed by other 50 ns of NVT MD simulation. The particle mesh Ewald (PME) method⁸ was used to treat the long-range electrostatics. For the calculation of the binding free energy between heparin and each compound, 5000 snapshots were saved during the MD data collection period described above, one snapshot per each 10 ps of MD simulation. All of the production molecular dynamics simulations were carried out working in our own CPU/GPU hybrid cluster.

To estimate the free energy of binding ΔG_{bind} , we resorted to a well-established computational recipe⁹ based on the MM/PBSA methodology.¹⁰ Briefly, the free energy of binding involved in the process may be generally written as $\Delta G_{\text{bind}} = G_{\text{AB}} - G_{\text{A}} - G_{\text{B}}$. For any species on the right hand side of this equation, from basic thermodynamics we have $G_i = H_i - TS_i$, where H_i and S_i are the enthalpy and entropy of the i-th species, respectively and T is the absolute temperature. In view of this expression, ΔG_{bind} can then be written as: $\Delta G_{\text{bind}} = \Delta H_{\text{bind}} - T\Delta S_{\text{bind}}$. ΔH_{bind} is the variation in enthalpy upon association and, in the MM/PBSA framework of theory, can be calculated by

summing the molecular mechanics energies (ΔE_{MM}) and the solvation free energy (ΔG_{solv}), i.e., $\Delta H_{bind} = \Delta E_{MM} + \Delta G_{solv}$. Finally, the estimation of the entropic contribution – T ΔS_{bind} is performed using normal mode analysis, which requires the computation of eigenvectors and eigenvalues via the diagonalization of the Hessian matrix.

We deconvoluted the enthalpic term of the ΔG_{bind} into its main components for each individual residues of HSA and β -CD-g-PG in the corresponding systems. This allowed us to determine the relative contribution of electrostatic binding and dispersion interactions to the overall binding event, and hence determine the dominant factors controlling the response of binding. This analysis was carried out using the MM/GBSA approach,¹¹ and was based on the same snapshots used in the binding free energy calculation.

In vitro drug release tests.

The release of drugs (DOX and PTX) from the β -CD-g-PG/HSA supramolecular complex was performed in a phosphate buffer solution (pH =7.4). β -CD-g-PG/HSA containing drugs (DOX or PTX) were loaded into dialysis bag (1000 MW) and immersed into drug-free 100 mL PBS buffer that was kept in a temperature controlled water bath. The release experiments were conducted at controlled temperature (37°C). During the release, 4 mL sample of medium was taken out at a desired period and subjected to UV-vis measurement. The quantitative analysis of drugs (DOX or PTX) was based on UV-vis data, since the intensity of the peaks (490 nm for DOX and 227 for PTX) is dependent on the concentration of each drug. By referring to a calibration curve prepared separately, the amount of released drugs (DOX or PTX) was determined after different time periods.

Drug loading efficacy (DLE).

The β -CD-g-PG/HSA complex loaded with each drug (DOX or PTX) was dialyses for 1h in phosphate buffer at pH=7.4 to remove unbound drug. Afterward, DLE values were calculated from the corresponding UV and calibration curves.



Figure 1S. ¹H NMR spectra (400 MHz, D₂O) of β -CD-g-PG.



Figure 2S.¹³C NMR spectra (100 MHz, D₂O) of β -CD-g-PG.



Figure 3S. Quenching of the fluorescence of β -CD-g-PG/HSA supramolecular complex by addition of paclitaxel (PTX) at 37°C and pH 7.4. Concentration of PTX from top to bottom: 1.4× 1.9, 2.4, 3.7, and 4.2×10⁻⁴ M. [HSA] =2 ×10⁻⁶ M. An ethanol/water (50:50) mixture was used as solvent.



Figure 4S. Quenching of the fluorescence of β -CD-g-PG/HSA supramolecular complex by addition of doxorubicin (DOX) at 37°C and pH 7.4. Concentration of PTX from top to bottom: 1.4×1.9 , 2.4, 3.7, and 4.2×10^{-4} M. [HSA] = 2×10^{-6} M. An ethanol/water (50:50) mixture was used as solvent.



Figure 5S. Far-UV CD spectra of HSA/DOX and HSA/PTX (right). Concentrations of DOX and PTX from a to c: 1.4, 2.8, and 4.2×10^{-4} M. All spectra were recorded in ethanol/water solution at 37 °C.

Table 1S. Amount of different secondary structural motifs in the HSA/DOX and HSA/PTX complexes as derived from the corresponding CD spectra. $[DOX] = [PTX] = 4.2 \times 10^{-4} \text{ M}.$

Structure	HSA/DOX	HSA/PTX
a-helix	52.10%	50.35%
β-antiparallel	5.56%	6.1%
β-sheet	4.23%	4.15%
β-turn	13.47%	14.3%
random coil	24.64%	25.1%



Figure 6S. Estimation of the binding constant K_b for β -CD-g-PG onto HSA from UV-vis data (Eq. (6)). Concentrations of HSA and β -CD-g-PG are the same as in Figure 6. T = 37°C.



Figure 7S. DLS diagrams for (**a**) HSA, (**b**) β-CD-g-PG, and (**c**) β-CD-g-PG/HSA complex.



Figure 8S. DLS diagrams for (a) HSA/DOX and (b) HSA/PTX.



Figure 9S. DLS diagrams for (a) HSA/ β -CD-g-PG/DOX, (b) HSA/ β -CD-g-PG/HSA/PTX, (c) HSA/ β -CD-g-PG/DOX/PTX complex.



Figure 10S. FT-IR spectra of (a) β -CD and (b) β -CD-g-PG.



Figure 11S. FT-IR spectra of (a) HSA and (b) HSA/ β -CD-g-PG complex.



Figure 12S. FT-IR spectra of (a) HSA/ β -CD-g-PG/DOX complex and (b) HSA/ β -CD-g-PG/PTX complex.



Figure 13S. Calibration curve for doxorubicin in chloroform.

DLE= (weight of loaded drug/weight of drug in feed) × 100 DLE=80% DLE of DOX/HSA=60% DLE of DOX /HSA-β-CD-g-PG=80% DLE of DOX /HSA (dialysis)=30% DLE of DOX /HSA-β-CD-g- PG (dialysis)=50%



Figure 14S. Calibration curve for paclitaxel in chloroform.

DLE= (weight of loaded drug/weight of drug in feed) \times 100 DLE=71% DLE of PTX/HSA=50% DLE of PTX/HSA- β -CD-g-PG=71% DLE of PTX/HSA (dialysis)=35% DLE of PTX/HSA- β -CD-g-PG (dialysis)=55%

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