Computational and Biological Evaluation of the Soybean Lecithin-Gallic Acid Complex for Ameliorating Alcoholic Liver Disease in Mice with Iron Overload

Xiangqun Wu[1], Yan Wang[2], Ran Jia[3], Fang Fang[1]*, Ya Liu[1]* and Weiwei Cui[1]*

[1] Department of Nutrition and Food Hygiene, School of Public Health, Jilin University, Changchun, P. R. China.

[2] Institute of Biomedical and Pharmaceutical Sciences, Guangdong University of Technology, Higher Education Mega Center, Guangzhou, P. R. China.

3 State Key Laboratory of Theoretical and Computational Chemistry, Institute of Theoretical Chemistry, Jilin University, Changchun, P. R. China.

***Corresponding Authors:**

*** Weiwei Cui:** Department of Nutrition and Food Hygiene, School of Public Health, Jilin University, Changchun, P. R. China.

*** Fang Fang:** Department of Nutrition and Food Hygiene, School of Public Health, Jilin University, Changchun, P. R. China.

*** Ya Liu:** Department of Nutrition and Food Hygiene, School of Public Health, Jilin University, Changchun, P. R. China.

Authors' Email Addresses:

Weiwei Cui: Tel: +86-431-8561-9455 E-mail: cuiweiwei@jlu.edu.cn

Fang Fang: Tel: +86-431-8561-9455 E-mail, fangfang7786@jlu.edu.cn

Ya Liu: Tel: +86-431-8561-9455 E-mail, liuya@jlu.edu.cn

Materials and Methods

Measurement of gallic acid content and complexing rate

A high-performance liquid chromatograph (Waters 600 HPLC, Waters Corporation, Milford, MA, US) was used to measure the gallic acid (GA) content in the eluate and to calculate the complexing rate. The chromatographic conditions were as follows: analytical HPLC was carried out with diode array detection at 283 nm on an Agilent HC-C18 column (250 mm × 4.6 mm, 5 μm) with isocratic elution in 80% acetonitrile/20% water (0.1% formic acid), column temperature at 25°C, and feeding of 20 μL.

Ultraviolet spectrum analysis

Solutions of samples to be measured were scanned at wavelengths of 200–400 nm for the ultraviolet spectrum analysis (UV-3600 ultraviolet-visible Spectrophotometer, Shimadzu, Japan).

Differential thermal analysis

The temperature was increased by 5°C every minute over a measurement range of 50°C–800°C, and GA, soybean lecithin, the soybean lecithin-GA complex (SL-GAC), and a physical mixture of soybean lecithin and GA were analyzed using differential scanning calorimetry (DSC; Heson, Shanghai, China).

Computational methods

1. Construction of lecithin and GA molecules. The atomic coordinates of the lecithin molecule were extracted from the crystal structure of human phosphatidylcholine transfer protein (hPTP; PDB ID: 1LN1). GA was built with the aid of Discovery Studio v4.0 Visualizer. Both molecules were optimized using the Gaussian v09 software package under the 6-311+g(d,p) basis set by the B3LYP method.[1] The optimized structures of the lecithin and GA molecules were used for the subsequent studies.

2. Generation of the structure of SL-GAC. Gaussian-accelerated molecular dynamics (GaMD) is a biomolecular enhanced-sampling method that works by adding a harmonic boost potential to smoothen the system potential energy surface.[2] Without the need to set predefined reaction coordinates, GaMD enables unconstrained enhanced sampling of biomolecules. This method can also be applied to sample the binding mode between two molecules, such as the lecithin and GA molecules. The structure of SL-GAC is unknown. It has been reported that in this complex, GA and lecithin combine via a non-covalent bond and do not form a new compound.[3] Therefore, we can employ GaMD to sample the non-covalent complex of lecithin and GA. The optimized structures of the lecithin and GA molecules were first placed randomly, and the centroid distance between these two molecules was approximately 50 Å. Then, these two molecules were solvated in an octahedral periodic box by using the TIP3P water model. The distance

between the outermost lecithin and GA atoms, and the walls of the simulation box was set to 10.0 Å. Then, GaMD simulation was performed for a total of 2 μs for the lecithin and GA systems, and this generated 100,000 frames. The GaMD simulation was conducted using the AMBER v16 software package.[4]

3. Construction of lecithin-GA-human phosphatidylcholine transfer protein complex. By performing GaMD on the lecithin and GA molecules, we obtained the non-covalent complex of lecithin and GA. To determine how this non-covalent complex interacted with the target protein, we used the molecular docking method to construct a complex structure of lecithin-GA-hPTP. The atomic coordinates of hPTP were extracted from its crystal structure (PDB ID: 1LN1). The lecithin molecule in this crystal structure was removed, and the non-covalent complex of lecithin and GA obtained from the GaMD simulation was docked into the active site of 1LN1. The molecular docking was conducted using the AutoDock v4.2 software package.[5] The nonpolar hydrogen atoms were removed, and only the polar hydrogens were retained. Gasteiger charges were added to the hPTP and non-covalent lecithin-GA complex. A box size of 40 × 40 × 40 $Å^3$ with a grid spacing of 0.375 Å was defined around the binding site of hPTP, so that it contained all the residues that are critical for interacting with the non-covalent complex. The grid map around the binding site of hPTP was generated using the probe atoms and the Auto Grid program. Each grid in the map represented the potential energy of a probe atom in the presence of all the atoms of the

receptor molecule. The Lamarckian genetic algorithm was used for the docking study. One hundred runs with 15,000,000 maximum evaluations and 270,000 generations were used for the docking simulation. The docking pose with the lowest binding energy (−5.67 kcal mol^{-1}) was chosen as the starting structure for the subsequent molecular dynamic (MD) simulation.

4. MD simulation of hPTP and lecithin-GA complex structure

Based on the complex structure obtained using the molecular docking, MD simulation was carried out. To determine how the lecithin-GA complex affected the structure of hPTP, we constructed two systems: an hPTP-only system that contained no ligand (denoted as apo-hPTP) and hPTP complexed with lecithin and GA (denoted as complex-hPTP). All MD simulations were carried out using the AMBER v16 software package.[4] All the missing hydrogen atoms of the hPTP protein were added using the LEaP module. The ff14SB force field was applied for the hPTP protein.[6] The parameter sets for the lecithin and GA molecules were supplied by the general AMBER force field.[7] Sodium ions were added to the complex systems by using a coulomb potential grid to keep the whole system neutral. According to the experimental process, the mice were fed an excess amount of alcohol. To simulate this condition, the apo-hPTP and complex-hPTP structures were solvated by water and ethanol molecules. The TIP3P water model and ethanol molecules were employed to solvate the two systems.[8] Both systems were solvated in an octahedral periodic box. The distance between the

outermost protein atoms and the walls of the simulation box was set to 10.0 Å. Each system was first submitted to 4000 steps of steepest-descent minimization and then to 6000 steps of conjugate-gradient minimization. Subsequently, the two systems were heated from 0 to 310 K in 500 ps. The heating process was conducted under the canonical ensemble (NVT ensemble). A harmonic restraint with a force constant of 10.0 kcal mol^{-1} $Å^{-2}$ was applied to the whole system. A Langevin thermostat was used during the heating process. Then, under an NPT ensemble with constant pressure (1.0 bar), the two systems were equilibrated for 5 ns. The relaxation time for the barostat bath was set to 2.0 fs. In the end, the apo-hPTP and complex-hPTP systems were both simulated for 200 ns. The NPT ensemble was used for this process, and periodic boundary conditions were employed. The long-range electrostatics was handled using the particle-mesh Ewald method.[9] The cut-off value for short-range interactions was set to 10.0 Å. The SHAKE algorithm was employed to constrain bonds involving hydrogen. The time step for all the simulations was set to 2 fs.

5. Calculation of binding free energies

The molecular mechanics/generalized Born surface area (MM/GBSA) method was implemented using AMBER v16 to calculate the binding free energy between SL-GAC and hPTP.[10, 11] The binding free energy ($^{\Delta G_{bind}}$) in MM/GBSA between a ligand (L) and a receptor (R) to form a complex RL was calculated as follows:

$$\Delta G_{bind} = G_{complex} - (G_{receptor} + G_{ligand}) \quad (1)$$

106 $$G = E_{MM} + G_{sol} - TS \quad (2)$$

107 $$E_{MM} = E_{int} + E_{ele} + E_{vdw} \quad (3)$$

108 $$G_{sol} = G_{GB} + G_{SA} \quad (4)$$

109 In eqn (2), E_{MM}, G_{sol}, and TS represent the molecular mechanics component in the gas
110 phase, the stabilization energy due to solvation, and a vibrational entropy term,
111 respectively. E_{MM} is given as the sum of E_{int}, E_{ele}, and E_{vdw}, which are internal, Coulomb,
112 and van der Waals interaction terms, respectively. The solvation energy, G_{sol}, is
113 separated into an electrostatic solvation free energy (G_{GB}) and a nonpolar solvation free
114 energy (G_{SA}). The former can be obtained using the Generalized Born (GB) method.
115 The latter is considered to be proportional to the molecular solvent accessible surface
116 area.[12] The binding free energies were obtained by averaging the values calculated for
117 5000 frames from the last 50 ns of the trajectories at 5-ps intervals for the complex
118 structure.
119
120 **Acute toxicity study**
121 The principles of laboratory animal care were followed, and all procedures were
122 conducted according to the guidelines established by the National Institutes of Health.
123 Every effort was made to minimize suffering. This study was approved by the Animal
124 Experiment Committee of Jilin University. A total of 20 healthy ICR mice (10 male,
125 10 female) weighing 20.0 ± 2.0 g were supplied by the Experimental Animal Center of
126 Public Health College, Jilin University (Jilin, China). All mice were accommodated

under the following conditions: room temperature, 25°C ± 2°C; relative humidity, 60% ± 10%; room air changes, 12–18 times/h; and a 12-h light/dark cycle. Throughout the study, the mice were fed a normal chow diet and purified water ad libitum. However, they were fasted for 6 h prior to the oral administration. The test was conducted according to OECD guidelines.[13] The animals were randomly divided into 2 groups of 10 mice each, including 5 animals of each sex. SL-GAC was suspended in purified water, and was administered at a dose of 5000 mg/kg bodyweight (BW) by gavage to the mice in the experimental group. The other group served as the control. After the administration of SL-GAC, the clinical symptoms of the mice were observed immediately after dosing, at 6 h, and at 24-h intervals, and then at 24-h intervals for 7 days. Clinical symptoms, including mortality, clinical signs, and gross findings, were recorded. On day 7, the mice were sacrificed by cervical dislocation and examined by necropsy.

Results

Ultraviolet spectrum analysis

From the ultraviolet (UV) diagrams for GA, soybean lecithin, SL-GAC, and soybean lecithin-GA physical mixture (Fig. S1), we found that lecithin did not have a characteristic absorption peak within the scope of the scanning wavelength. GA had its maximum absorption peak at a wavelength of 272 nm. The physical mixture and the complex also had maximum absorption peaks with the same wavelengths, but the peak of the complex was not as high as that of GA at the same concentration. This proved that the complex had not resulted in any changes to the chemical structure of GA.

Furthermore, the complex mainly showed the chemical properties of GA. The decrease in its characteristic peak may be because the characteristic structure was involved in the SL-GAC.

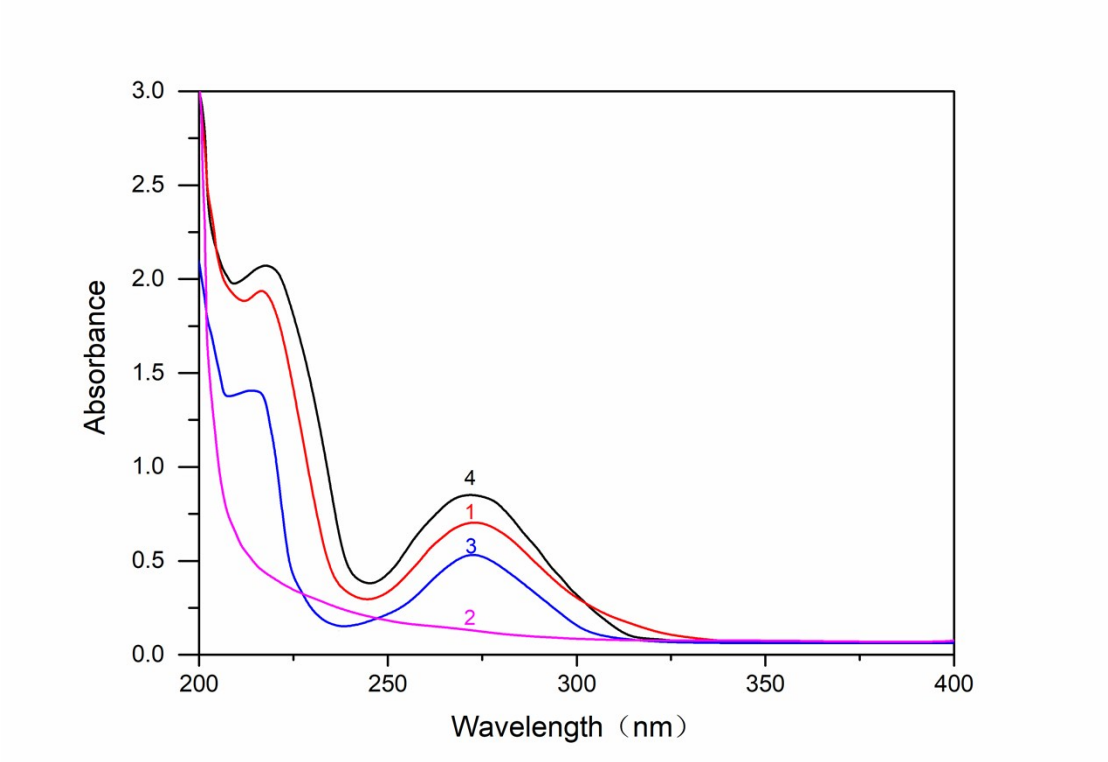
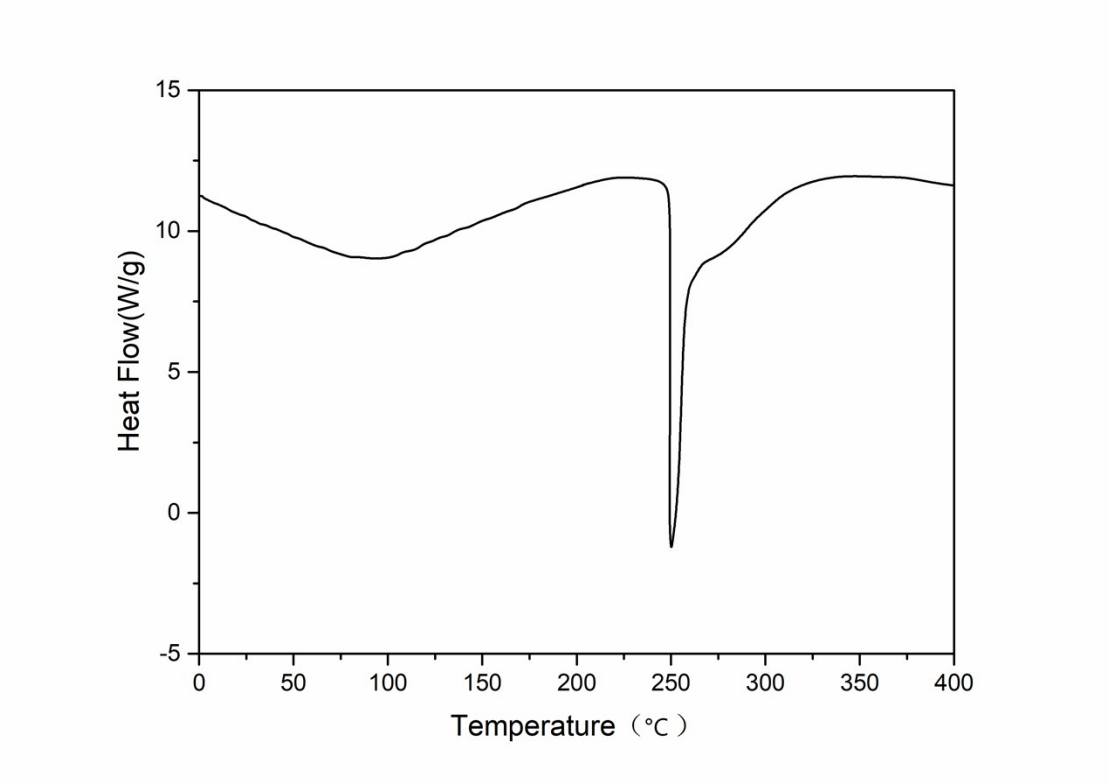


Figure S1. Ultraviolet spectrum curves of GA (1), soybean lecithin (2), SL-GAC (3), and the physical mixture of gallic acid and soybean lecithin (4)

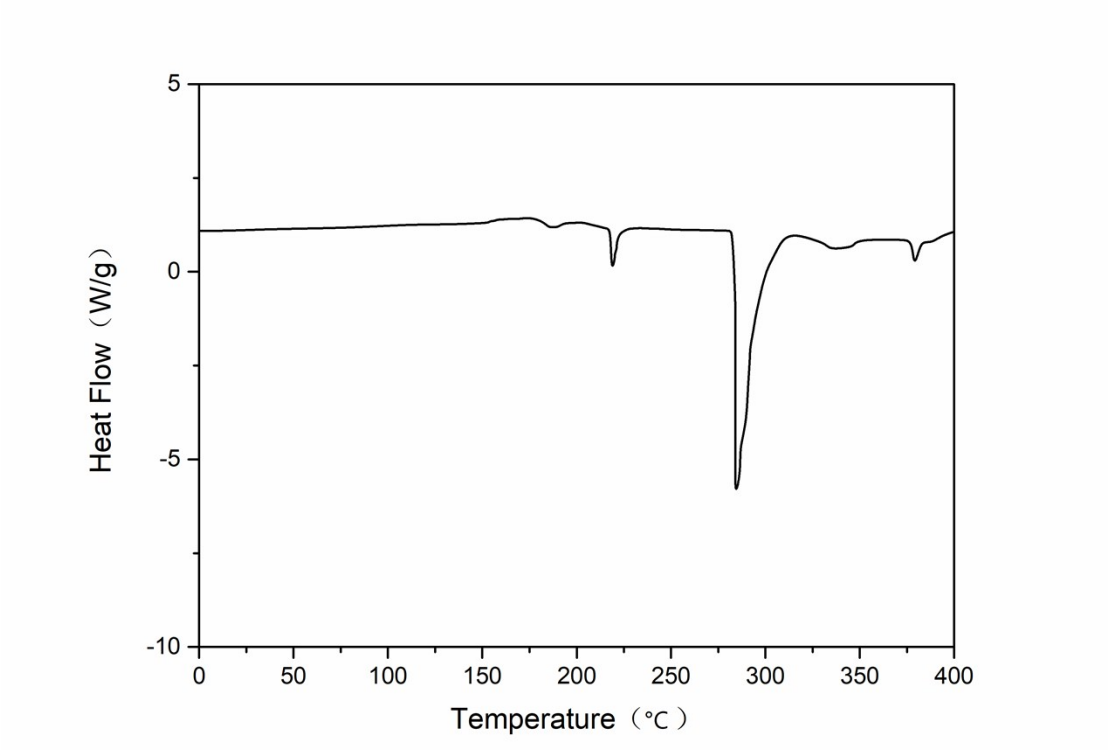
Differential thermal analysis

As indicated by the differential scanning calorimeter (DSC) diagram of GA, soybean lecithin, and their mixture and complex (Fig. S2–S5), GA began to have an absorption peak at 250°C, which corresponds to its melting point (Fig. S2). With an amorphous form, soybean lecithin had no definite melting point and had a large absorption peak at 276°C and two small absorption peaks at 207.2°C and 381.5°C each

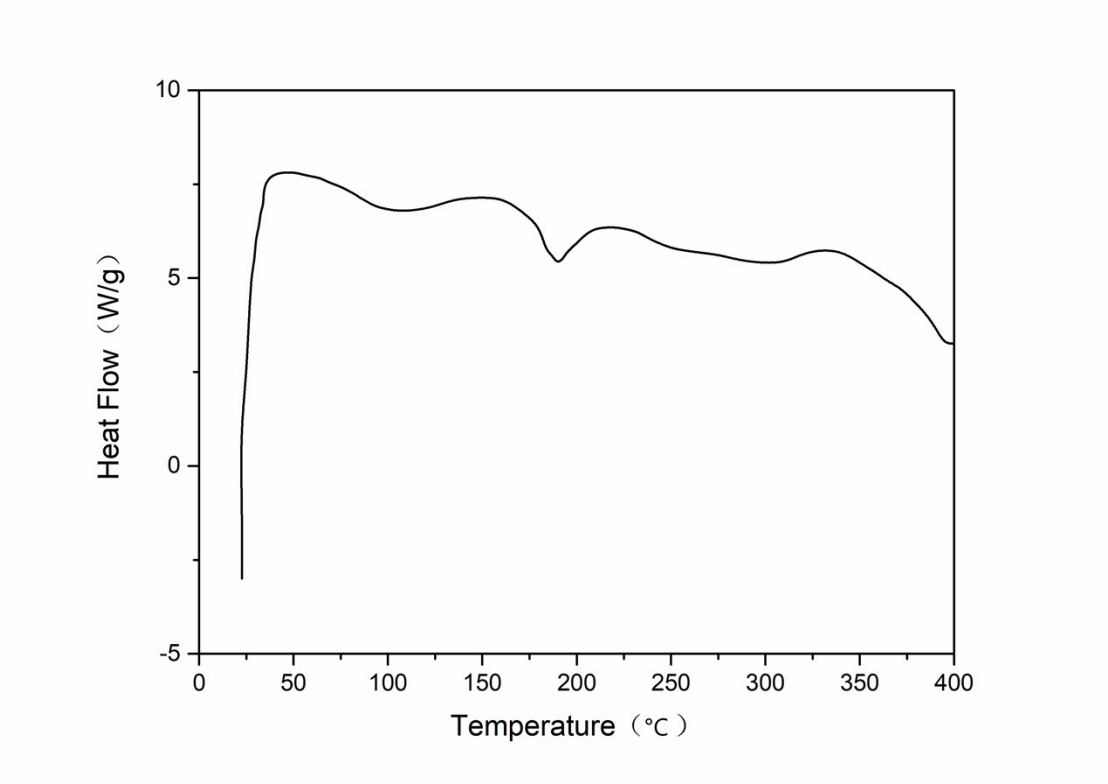
(Fig. S3). The small peak at 207.2°C may have been caused by the polar end of lecithin, whereas the latter two peaks were caused by a change in the physical phase from gel to liquid as the long chain between carbon and hydrogen was broken or separated. The melting peak of GA in the complex disappeared completely, and in general, its DSC diagram was very similar to that of lecithin; however, the heat absorption peak appeared at a lower temperature, which indicated that GA no longer existed in the form of crystals, and was instead completely dispersed in lecithin (Fig. S4). The reason for this is probably because the polar ends of the GA and lecithin molecules were combined, which degraded the orderliness among the aliphatic hydrocarbon chains of lecithin. The DSC diagram of the GA-soybean lecithin physical mixture mainly showed a superposition of the diagrams of the two molecules, and the melting peak of GA could still be observed (Fig. S5).



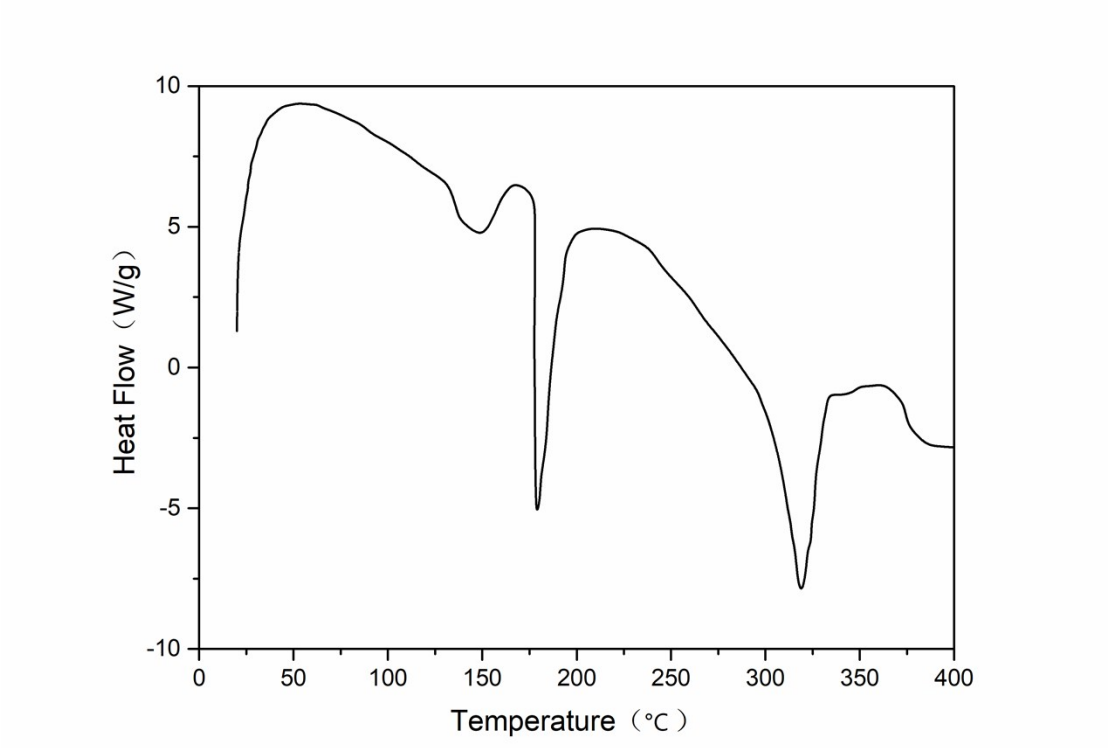
175 **Figure S2.** Differential scanning calorimetry curve of GA



176 **Figure S3.** Differential scanning calorimetry curve of soybean lecithin



177 **Figure S4.** Differential scanning calorimetry curve of SL-GAC



178 **Figure S5.** Differential scanning calorimetry curve of the physical mixture of GA and
179 soybean lecithin
180

Computer simulations

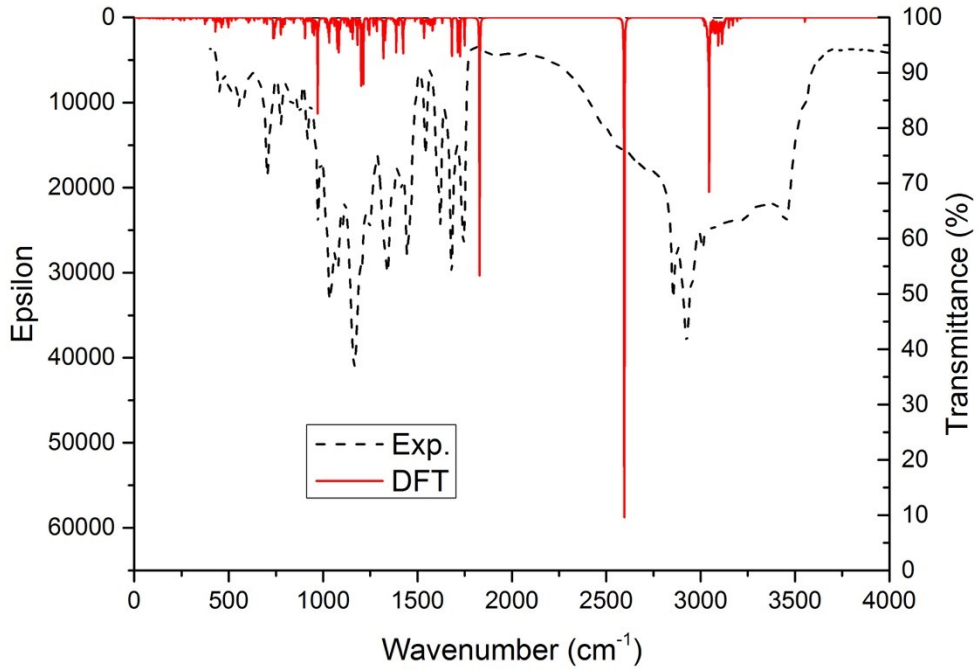
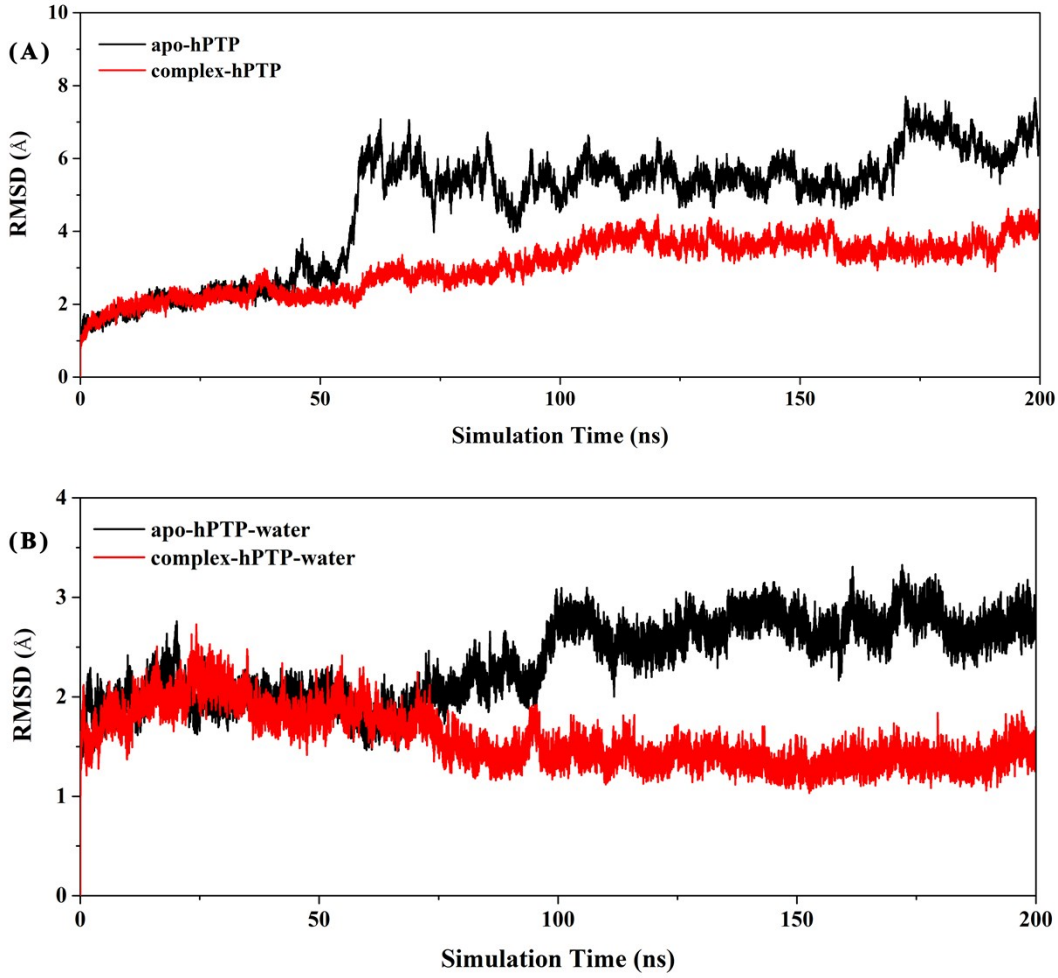


Figure S6. Computational infrared (IR; red lines) and experimental IR (black dashed lines)



184 **Figure S7.** Root means square deviation (RMSD) plots for apo-hPTP and complex-
185 hPTP in both (A) ethanol and (B) water
186

Table S1. Hydrogen-bond analysis between the hydroxyl group of GA and the phosphate group of lecithin

Donor	Acceptor	Average distance (Å)	Average angle (°)	Percentage (%)
O5@lecithin	H2@gallic acid	1.64	166.77	100
O5@lecithin	H3@gallic acid	1.68	165.42	100

Table S2. Binding free energies (kcal·mol^{-1})

	ΔG_{VDW}	ΔG_{ele}	ΔG_{GB}	ΔG_{SURF}	ΔG_{TOT}	$T\Delta S$	$\Delta G_{binding}$
Ethanol	-102.60	-31.80	69.00	-14.70	-80.098	-48.55	-31.55 ± 8.98
Water	-108.37	-51.55	88.61	-15.25	-86.56	-38.88	-47.68 ± 7.85

Acute toxicity of SL-GAC

The clinical symptoms of mice were observed for 7 days after the intragastric administration of SL-GAC. All of the mice administered SL-GAC at a dose of 5000 mg/kg survived the 7-day observation period. There were no clinical signs of toxicity throughout the experimental period. The administration of SL-GAC did not cause any appreciable alteration in the mean body weights of the mice.

To determine the safety of SL-GAC, we performed an oral acute toxicity test. The absence of any adverse e□ects after the administration of a dose of 5000 mg/kg clearly indicated the non-toxic nature of SL-GAC. Toxicologists agree that any test substance that is not lethal when administered as a single oral dose at a concentration of 5000 mg/kg is essentially non-toxic.[13, 14] Therefore, it may be concluded that SL-GAC was practically non-toxic and was safe via the oral route.

Table S3. Body weight of SL-GAC-fed mice in the acute toxicity study (g/mouse)

	Time (day)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Control	18.7 ± 0.8	20.4 ± 1.1	20.7 ± 1.5	21.1 ± 1.8	21.7 ± 2.2	22.3 ± 2.6	22.9 ± 2.8
SL-GAC	17.2 ± 1.1	18.4 ± 1.2	19.2 ± 1.3	20.0 ± 1.5	20.8 ± 1.6	21.7 ± 1.7	22.6 ± 2.1

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