Supporting Information

Carbonization of quercetin into nanogels: a leap in anticoagulant development

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Supplementary experimental section

Chemicals. Quercetin was purchased from Cayman (Ann Arbor, MI, USA). Sodium phosphate, calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), potassium chloride (KCl), sodium chloride (NaCl), trisodium citrate, human α-thrombin (1,000 NIH units/mg protein), human fibrinogen, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Warfarin was purchased from Fluka (St. Muskegon, MI, USA). Enoxaparin was purchased from Merck (Darmstadt, Germany). Reagents for the measurement of prothrombin time (PT) and activated partial thromboplastin time (aPTT) were purchased from Helena Laboratories (Beaumont, TX, USA). Fisher Scientific Ultrapure water from a Mili-Q Simplicity^{TM} 185 system with a resistivity of no less than 18.2 MΩ.cm was used for all experiments.

Characterization of $CNGs_{Qur}$. Transmission electron microscopy (TEM) images of $CNGs_{Qur}$ were measured using a Tecnai G2 F20 S-TWIN (Philips/FEI, Hillsboro, OR, USA) system operating at 200 kV. $CNGs_{Qur}$ (20 μg mL^{-1}) were carefully deposited onto 300-mesh carbon-coated Cu grids, and excess solvent was evaporated at ambient temperature and pressure. A Synergy^{TM} 4 multimode microplate spectrophotometer (Biotek Instruments, Winooski, VT, USA) was used to measure the absorption of the $CNGs_{Qur}$ in ultrapure water. The fluorescence spectra of as-prepared $CNGs_{Qur}$ were recorded using a Synergy^{TM} 4 multimode microplate spectrophotometer at excitation wavelengths in the range 325–445 nm. The hydrodynamic size and zeta potentials (ζ) of the $CNGs_{Qur}$ were measured by a Zetasizer (Nano ZS, Malvern Instruments, Worcestershire, UK). Elemental analysis (EA) was performed using a Vario EL cube analyzer (Elementar, Hanau, Germany) for C, H, and O. Samples for X-ray diffraction (XRD) were prepared by depositing the $CNGs_{Qur}$ solution on the silicon wafer and drying at 50 ^{o}C for 12 h. XRD measurements were carried out at room temperature using an X-ray diffractometer (D/MAX 2200 VPC, Rigaku, Sendagaya, ShibuyaKu, Tokyo, Japan) with the Cu $K_{\alpha 1}$ line (λ = 1.54 Å, energy = 8.8 keV). A Fourier transform infrared spectrometer (FT-IR,

FT/IR-6100, JASCO, USA) in transmission mode in the range of 500 to 4000 cm^{-1} with 32 scans was used to identify the possible functional groups existing in the $CNGs_{Qur}$. High-purity nitrogen was used for purging during the FT-IR measurements to minimize the interference of water vapor. X-ray photoelectron spectroscopy (XPS) measurements were performed using an ESCALAB 250 spectrometer (VG Scientific, East Grinstead, UK) with Al Kα X-ray radiation as the X-ray source for excitation. Binding energies were corrected using the C 1s peak at 284.6 eV as the standard. Thermogravimetric analysis (TGA) of the $CNGs_{Qur}$ was conducted using a TGA instrument (Q500, TA Instruments, New Castle, DE, USA). Differential scanning calorimetry (DSC) measurements were performed with a TA 2010 instrument (TA Instruments LLC).

Determination of dissociation constant of thrombin–$CNGs_{Qur}$. To ascertain the dissociation constant (K_d) of $CNGs_{Qur}$ and thrombin, varying concentrations of thrombin (0–750 pM) and $CNGs_{Qur}$ (0.02 mg mL^{-1}) were allowed to react for 30 minutes before being separated using a size exclusion chromatography (SEC) column (Sephacryl S-200 HR; GE Healthcare Bioscience; Buckinghamshire, UK) with a bed volume of 1.5 mL. The sample solution was eluted 12 times with 0.1 mL of PBS solution, and each fraction was collected and labeled sequentially. Fractions 7, 8, 9, 10, 11, and 12 (containing unbound or free thrombin) were then combined with fibrinogen-modified gold nanoparticles (Au NPs; 56 nm) solution, reacted for 30 minutes, and the solution was centrifuged (500 g, 10 minutes). The absorbance of the supernatant was measured at 532 nm [40]. Free thrombin could catalyze the fibrinogen on Au NPs to form fibrin-Au NP aggregates. The dissociation constant K_d of $CNGs_{Qur}$ and thrombin was calculated using the Scatchard equation (1):

$$B_{Thrombin}/[\text{free thrombin}] = N_{max}/K_d - [\text{bound thrombin}]/K_d \quad (1)$$

Here, [bound thrombin] represents the concentration of thrombin bound to $CNGs_{Qur}$ at equilibrium, N_{max} denotes the maximum number of binding sites on $CNGs_{Qur}$, [free thrombin] refers to the unbound thrombin concentration at equilibrium, and K_d is the dissociation constant.

The K_d and N_{max} values were obtained from the slope and intercept, respectively, of the linear plot of $B_{Thrombin}$/[free thrombin] versus [bound thrombin].

Prothrombin time (PT) and activated partial thromboplastin time (aPTT). PT and aPTT reagents were utilized to evaluate the anticoagulation effect of CNGs on the extrinsic and intrinsic pathways, respectively. For the PT assessment, human plasma (200 μL) and inhibitor (20 μL) were pre-incubated at 37 °C for 3 minutes, followed by the addition of PT reagent (120 μL) to initiate the extrinsic coagulation cascade. Coagulation was determined by monitoring changes in scattered light intensity. In the aPTT assessment, human plasma (160 μL) and inhibitor (20 μL) were pre-incubated at 37 °C for 3 minutes, after which the aPTT reagent (95 μL) was added and incubated for an additional 3 minutes. Finally, calcium chloride ($CaCl_2$; 100 μL, 25 mM) at 37 °C was introduced to the mixture to trigger the intrinsic coagulation cascade, with coagulation determined by observing alterations in scattered light intensity. To calculate the PT and aPTT values, the endpoint was determined as the point where the scattered light signal reached halfway between its minimal and maximal values.

Coagulation factor activity assay. Coagulation factor activities (V, VII, X, XI, XII) were assessed using a Sysmex CS2500 System (Siemens Healthineers, Erlangen, Germany). $CNGs_{Qur}$ (2.0 mg mL^{-1}; 100 μL) was mixed with human plasma (900 μL) in vials. With the exception of the factor being measured, all other coagulation factors were added in excess, and the activation agents (20 μL) were introduced immediately prior to analysis. The clotting time for each factor was determined by monitoring the transmitted light (absorbance) at 660 nm. The raw data was then processed through a mathematical algorithm provided by the instrument to calculate the coagulation time. All assays were performed in accordance with the manufacturer's instructions and with the support of the National Taiwan University Hospital. Each test was conducted in triplicate from a single batch.

*Cytotoxic assay***.** Human lung adenocarcinoma epithelial cells (A549 cell line), human colon epithelial cells (HCT-116 cell line), human umbilical cord vein endothelial cells (HUVEC cell

line), human breast epithelial cells (MCF-7 cell line), and human non-small cell lung cancer epithelial cells (H1299 cell line) were obtained from the American Type Culture Collection Center (ATCC, Manassas, VA, USA). The A549, HCT-116, H1299, and MCF-7 cell lines were maintained in DMEM containing 10% fetal bovine serum (FBS), while HUVECs were routinely cultured in Vasculife EnGS ECG. All cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. Initially, the cells were seeded in 48-well plates and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. Then, the medium was removed, followed by washing with fresh medium. Subsequently, $CNGs_{Qur}$ prepared in the corresponding medium at varying concentrations was added to the wells, and the cells were incubated for an additional 24 hours. After removal of the supernatant, PrestoBlue was introduced, followed by incubation at 37°C for 1.5 hours. Fluorescence was then measured using a microplate spectrophotometer (Synergy 4, Biotek Instruments, Winooski, VT, USA) at an excitation and emission wavelength of 540 and 590 nm, respectively.

Hemolysis assay. Blood from healthy volunteers was collected using evacuated blood tubes containing ethylenediaminetetraacetic acid (EDTA), adhering to relevant laws and institutional guidelines. Red blood cells (RBCs) were obtained after centrifugation (3,000 *g*) at 4 °C for 10 minutes, followed by dilution using a sterile physiological buffer solution to prepare a 4% RBC stock suspension. This suspension was then mixed with varying concentrations of $CNGs_{Qur}$ (0–0.2 mg mL^{-1}) in PBS and incubated at 37 °C with shaking at 160 rpm for 1 hour. After incubation, each vial underwent centrifugation at 3,000 *g* at 4 °C for 10 minutes. Hemolytic activity was assessed by measuring the hemoglobin absorption at 576 nm ($OD_{576/CNGsQur}$) in the supernatant. A sterile isotonic physiological buffer served as a reference for 0% hemolysis ($OD_{576/blank}$), while the 100% hemolysis control was created by adding ultrapure water to the RBC suspension ($OD_{576/ultrapure\ water}$). Hemolytic activity was then calculated accordingly (2).

$$\text{Hemolysis (\%)} = [(OD_{576/CNGsQur} - OD_{576/blank}) / (OD_{576/ultrapure\ water} - OD_{576/blank})] \times 100 \quad (2)$$

Table S1. Yield, hydrodynamic diameter, elemental analysis of quercetin, PQur, $CNGs_{Qur230}$, $CNGs_{Qur250}$, $CNGs_{Qur270}$, $CNGs_{Qur290}$, and $CNGs_{Qur310}$.

	Yield (%)	Hydrodynamic diameter (nm)[a]	Elemental composition		
			C (%)[b]	H (%)[b]	O (%)[b]
quercetin	-	-	53.3	4.1	42.6
PQur	-	567.2 ± 28.3	41.3	3.9	54.7
$CNGs_{Qur230}$	82.5	1887.4 ± 683.8	36.9	3.1	59.8
$CNGs_{Qur250}$	80.0	1745.0 ± 295.0	38.3	3.9	57.6
$CNGs_{Qur270}$	80.0	1627.0 ± 299.8	40.1	4.0	55.7
$CNGs_{Qur290}$	32.5	1574.8 ± 251.7	45.5	3.4	50.8
$CNGs_{Qur310}$	22.5	1492.5 ± 90.6	-[c]	-[c]	-[c]

[a] Determined from three measurements

[b] Elemental composition was determined by elemental analysis.

[c] No data due to high material loss after dialysis.

Table S2. *R*, *K*, α Angle, and MA values in thrombin-triggered thromboelastography.

	R (min)	*K* (min)	*α* Angle (deg)	MA (mm)
Control	0.6 ± 0.1	1.6 ± 0.2	70.9 ± 1.1	60.8 ± 2.4
Quercetin	0.6 ± 0.2	1.4 ± 0.1	71.7 ± 2.3	51.5 ± 3.6
PQur	0.7 ± 0.1	2.5 ± 0.1	61.3 ± 3.1	51.0 ± 2.5
$CNGs_{Qur230}$	1.1 ± 0.3	2.2 ± 0.2	62.5 ± 2.4	53.6 ± 1.4
$CNGs_{Qur250}$	1.4 ± 0.2	3.2 ± 0.1	23.6 ± 2.6	29.2 ± 1.9
$CNGs_{Qur270}$	2.7 ± 0.3	11.4 ± 0.2	23.6 ± 3.3	29.2 ± 2.7
$CNGs_{Qur290}$	> 30.0	N/A	N/A	N/A
$CNGs_{Qur310}$	> 30.0	N/A	N/A	N/A
Enoxaparin	> 30.0	N/A	N/A	N/A

The standard deviations were calculated from five repeated experiments. N/A: not available.

Table S3. *R*, *K*, α Angle, and MA values in Kaolin-triggered thromboelastography.

	R (min)	*K* (min)	*α* Angle (deg)	MA (mm)
Control	5.8 ± 0.1	1.1 ± 0.1	72.0 ± 2.2	64.7 ± 3.5
Quercetin	10.6 ± 0.3	1.9 ± 0.2	61.8 ± 2.6	60.4 ± 6.1
PQur	12.8 ± 0.2	3.4 ± 0.3	55.6 ± 4.6	54.9 ± 5.6
$CNGs_{Qur230}$	15.4 ± 0.2	3.2 ± 0.2	49.0 ± 3.0	54.9 ± 4.7
$CNGs_{Qur250}$	19.8 ± 0.4	5.1 ± 0.2	35.8 ± 2.2	43.1 ± 3.8
$CNGs_{Qur270}$	> 30.0	N/A	N/A	N/A
$CNGs_{Qur290}$	> 30.0	N/A	N/A	N/A
$CNGs_{Qur310}$	> 30.0	N/A	N/A	N/A
Enoxaparin	> 30.0	N/A	N/A	N/A

The standard deviations were calculated from five repeated experiments. N/A: not available.

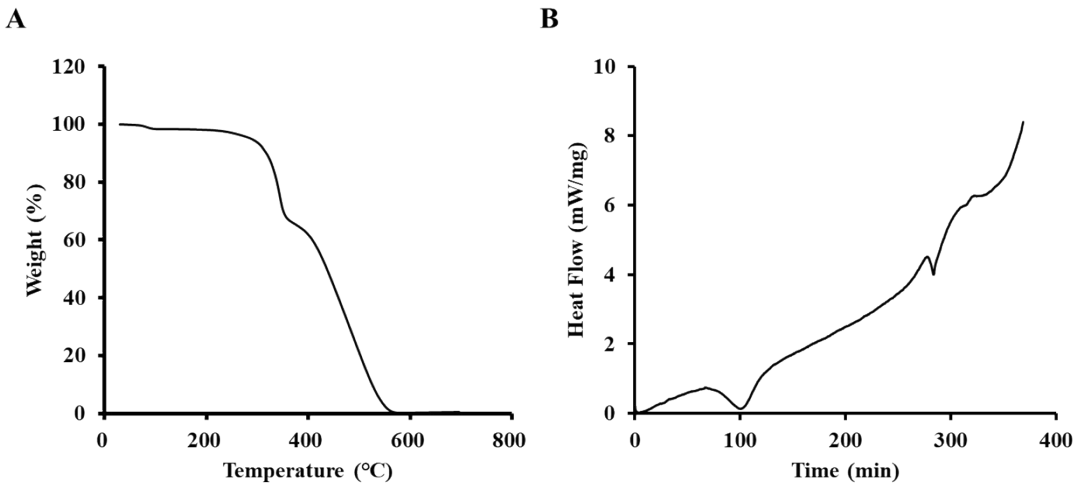

Fig. S1. (A) TGA and (B) DSC curves of quercetin in ambient atmosphere. The heating rate is set at 10 °C min^{-1}.

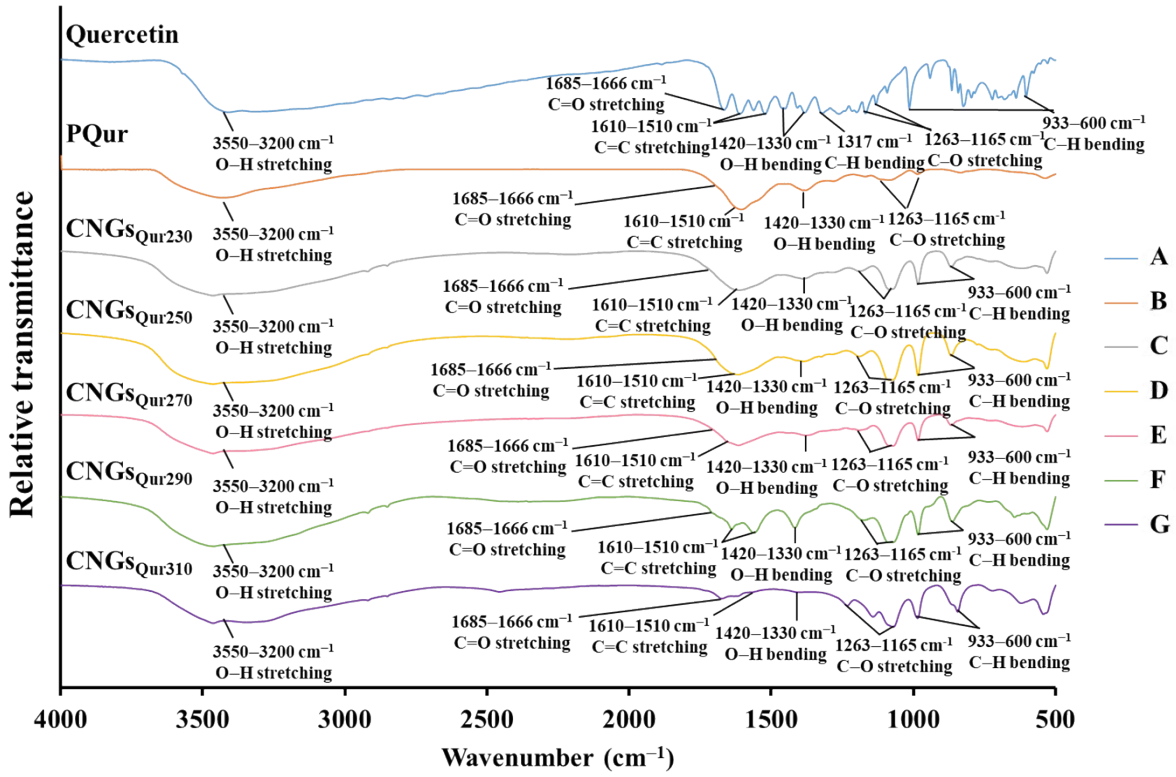

Fig. S2. FT-IR spectra of (A) quercetin, (B) PQur, (C) $CNGs_{Qur230}$, (D) $CNGs_{Qur250}$, (E) $CNGs_{Qur270}$, (F) $CNGs_{Qur290}$ and (G) $CNGs_{Qur310}$.

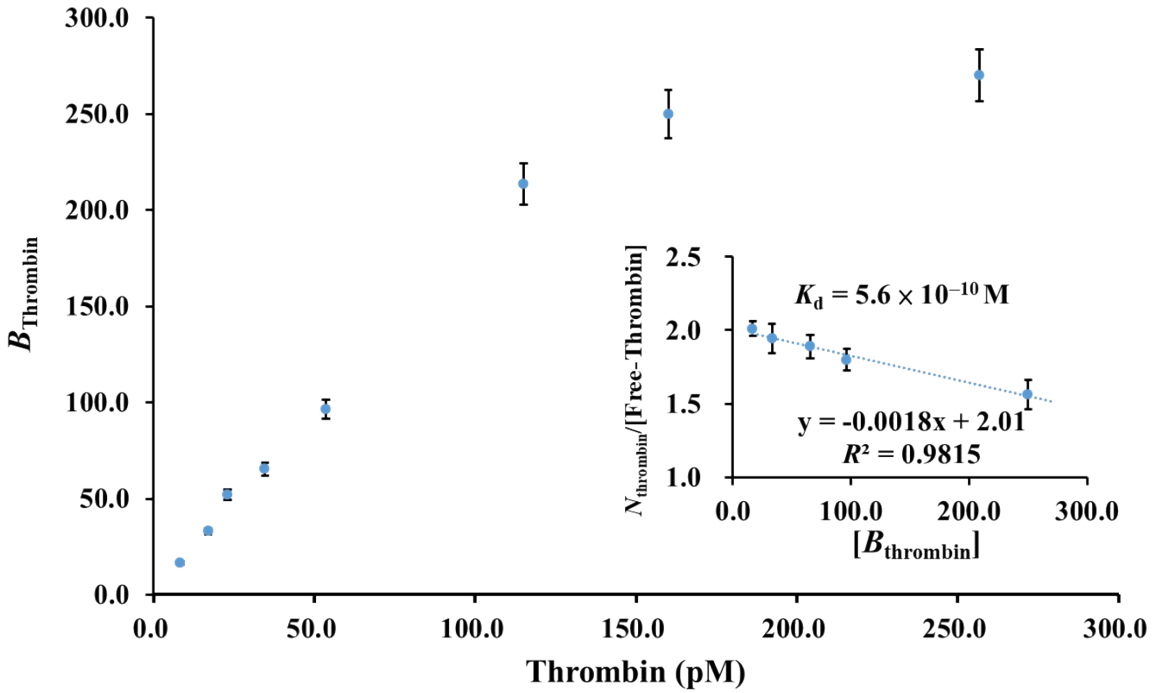

Fig. S3. Plot for calculating the dissociation constant (K_d) for thrombin and $CNGs_{Qur270}$. [$B_{Thrombin}$] is the concentration of thrombin molecules bound to $CNGs_{Qur270}$ at equilibrium and [Free-Thrombin] is the free thrombin concentration at equilibrium. The error bars represent the standard deviations of experiments in triplicate.

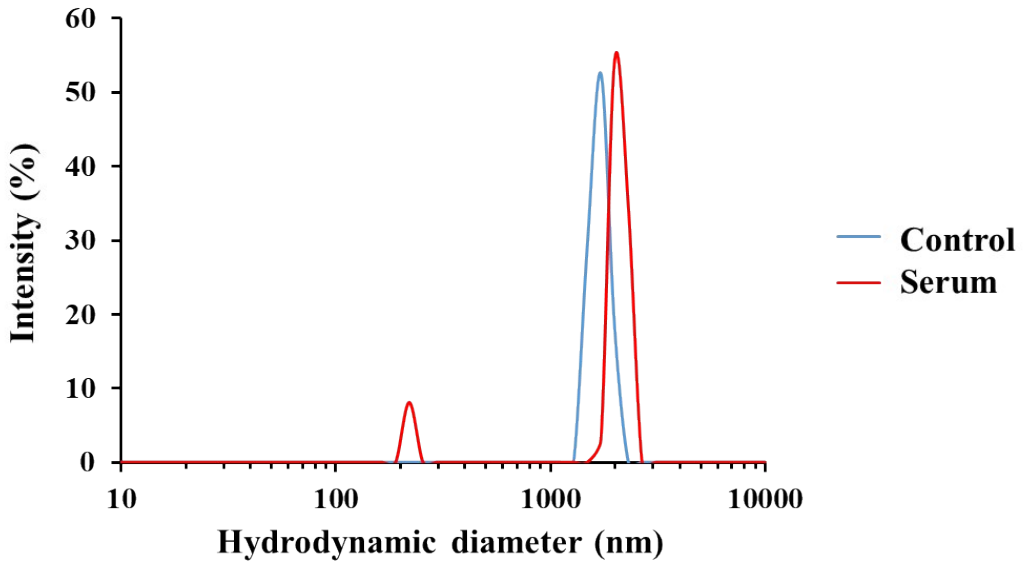

Fig. S4. DLS measurement of CNG_{Qur270} prepare in 5.0 sodium phosphate buffer (pH 7.4) as a control and after 24 h incubation in 2-fold diluted mice serum.

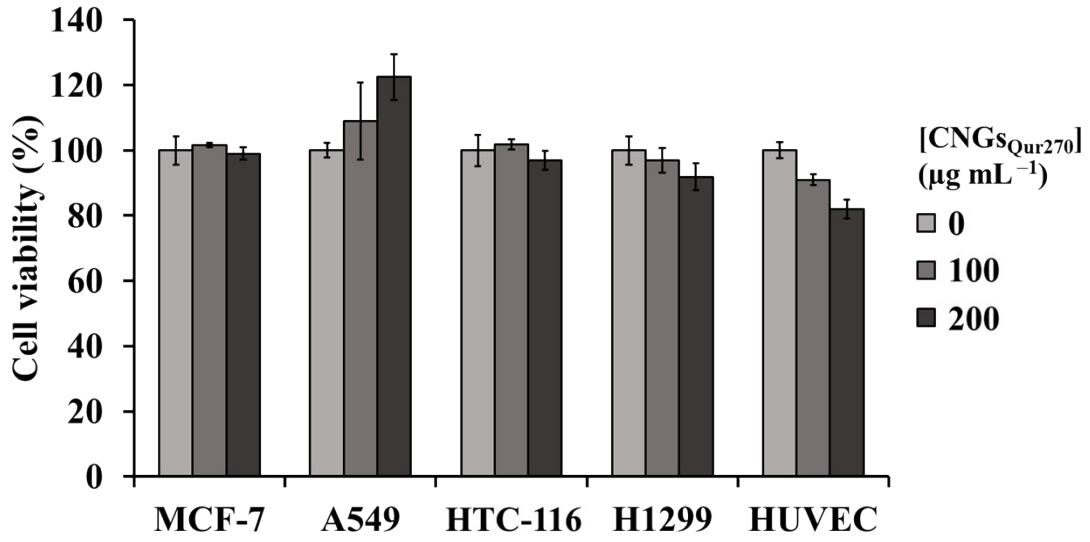

Fig. S5. Relative cell viability of MCF-7, A549, HCT-116, H1299, and HUVEC cell line after being treated with different concertation of $CNGs_{Qur270}$ for 48 h. The error bars represent the standard deviation of the values obtained from four repeated experiments.

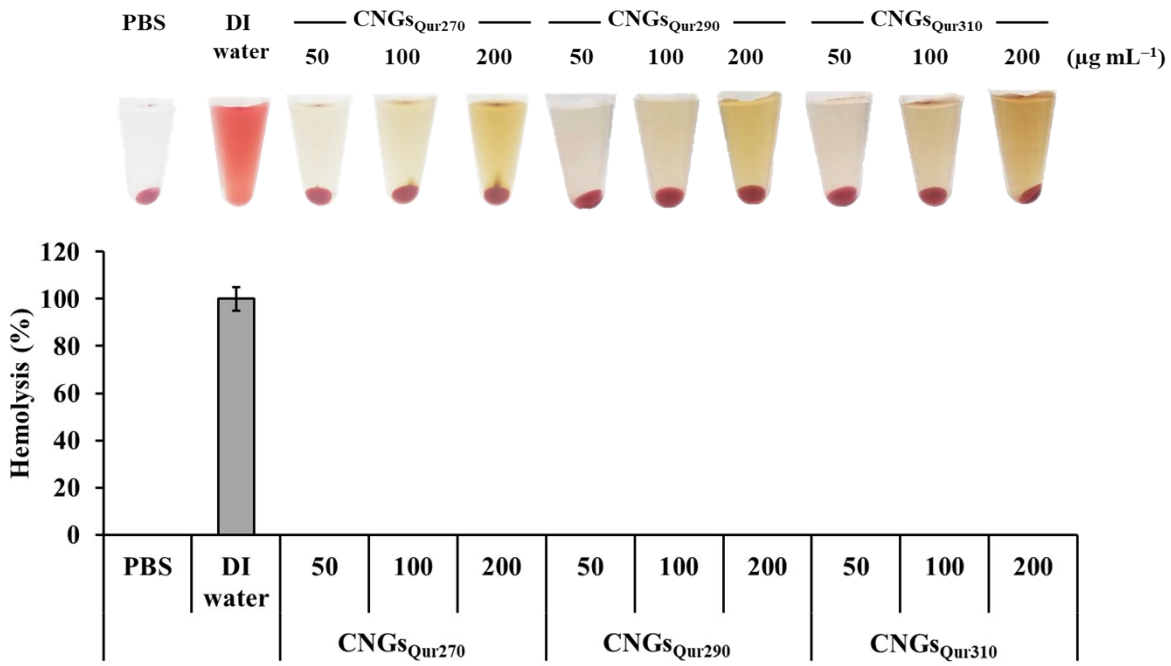

Fig. S6. Hemolysis assays of $CNGs_{Qur}$ at different concentrations. Error bars represent the standard deviation from four repeated measurements.

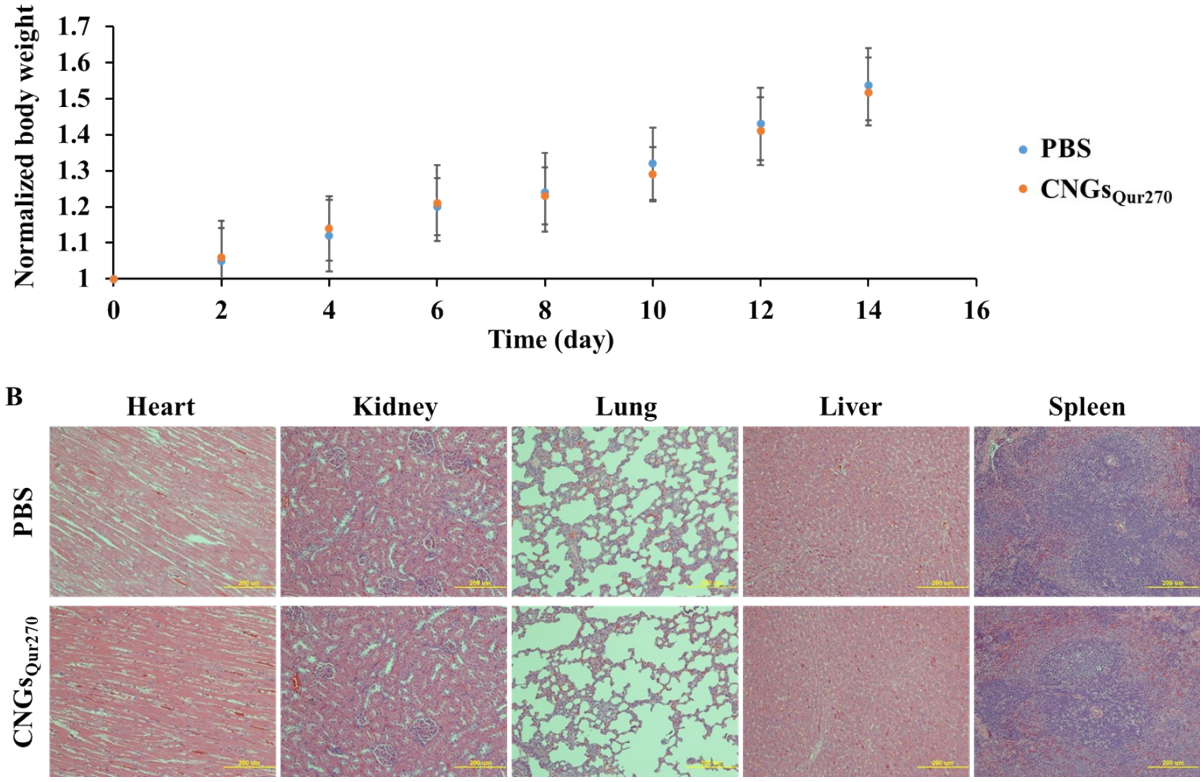

Fig. S7. (A) The normalized body weight of male Sprague-Dawley rats for 14 days, and (B) H&E staining of the tissues excised of male Sprague-Dawley rats at day 15 after the intravenous (IV) injection with PBS and $CNGs_{Qur270}$ (25 mg kg^{-1}). Bodyweight is determined and normalized according to the following equation: normalized body weight = W_n/W_0 (where W_n and W_0 are the body weights of the rats on Day n and Day 0, respectively). Error bars represent the standard deviations of experiments consisting of measurements for three rats.