Supplementary Information

Thermal-driven formation of polyphenolic carbonized nanogels with high anticoagulation activity from polysaccharides

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Experimental Section

Materials

Alginic acid, sodium salt (named as sodium alginate; >98%) was purchased from ACROS (Geel, Belgium). Fucoidan was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Prothrombin time (PT) and activated partial thromboplastin time (aPTT) assay reagents were purchased from Helena Laboratories (Beaumont, TX, USA). Sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (H_2NaPO_4), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), potassium chloride (KCl), sodium chloride (NaCl), trisodium citrate, human α-thrombin (1,000 NIH units mg^{-1} protein), bovine serum albumin (BSA), warfarin, rivaroxaban, dabigatran, and enoxaparin (low molecular weight heparin; MW is approximately 5,000 Da) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Biowest (Lewes, England, UK). Antibiotic antimycotic solution (100X), L-glutamine (200 mM), nonessential amino acids solution (100X), sodium pyruvate (100 mM), Dulbecco's Modified Eagle's medium (DMEM), medium 199 (M199), Kaighn's modification of Ham's F-12 medium (F-12K), trypsin-EDTA solution (0.25%), sodium bicarbonate (99.5–100.5%), PrestoBlue cell viability reagent, LIVE/DEAD Viability/Cytotoxicity Kit, and all cell culture media were purchased from Invitrogen (Eugene, OR, USA). Physiological buffer solution (PBS; 25.0 mM Tris-HCl, 150.0 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$ and 1.0 mM $CaCl_2$; adjusted to pH 7.4 using HCl) was used to mimic physiological conditions. Ultrapure water (18.2 MΩ cm) from a Milli-Q ultrapure water system (Millipore, Billerica, MA, USA) was used for all experiments.

Characterization of GNS/Alg-NGs

The particle size and morphology of the GNS/Alg-NGs were analyzed using a Tecnai G2 F20 S-TWIN (Philips/FEI, Hillsboro, OG, USA) transmission electron microscopy (TEM) system operating at 200 kV. The as-prepared GNS/Alg-NGs were carefully deposited on 300-mesh carbon-coated Cu grids, at ambient temperature. The hydrodynamic diameters and zeta potential (ζ-potential) of untreated sodium alginate and GNS/Alg-NGs in 5 mM sodium phosphate buffer (pH 7.4) was determined by dynamic light scattering (DLS, Zetasizer Nano ZS90, Malvern Instruments, Malvern, UK). The photoluminescence (PL) spectra of the as-prepared GNS/Alg-NGs dispersions in 5 mM sodium phosphate buffer (pH 7.4) were recorded

using a monochromatic microplate spectrophotometer (Synergy 4 Multi-Mode; BioTek Instruments, Winooski, VT, USA). The fluorescence quantum yields (QYs) of the GNS/Alg-NGs were determined by comparison with that of quinine sulfate standard (QY = 54% in 0.1 M H_2SO_4). Samples for X-ray diffraction (XRD) were prepared by depositing the GNS/Alg-NGs solution onto a silicon wafer and followed by drying at room temperature. XRD measurements were carried out at room temperature with 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). Elemental analysis (EA) of the GNS/Alg-NGs was performed using a Vario EL cube analyzer (Elementar, Hanau, Germany) for C, H, and O. X-ray photoelectron spectroscopy (XPS) was performed using an ESCALAB 250 spectrometer (VG Scientific, East Grinstead, England, UK) with Al Kα X-ray radiation as the X-ray source for excitation. Binding energies were corrected using the C1s peak at 284.6 eV as the standard. A Fourier transform infrared spectrometer (FT-IR, FT/IR-6100, JASCO, Easton, MD, USA) in transmission mode in the range of 500 to 4,000 cm^{-1} with 16 scans was used to analyze possible functional groups existing in the GNS/Alg-NGs. High-purity nitrogen was used for purging during the FT-IR measurements to minimize the interference from water vapor. Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) of the as-prepared materials was conducted using a TGA instrument (Q500, TA Instruments, New Castle, DE, USA) and a TA 2010 instrument (TA Instruments) in an air atmosphere (60 mL min^{-1}), respectively. The viscosity was measured with a cone plate (cone spindle: CPA-40Z) viscometer (Brookfield, model DV1MLV, Middleboro, MA, USA). Cyclic voltammetry (CV) was performed in PBS solution by using a three-electrode system composed of a glassy carbon electrode (GCE) as the working electrode, a platinum electrode as the counter electrode, and a saturated calomel electrode (SCE) as a reference electrode. Cyclic voltammetry was conducted using a potentiostat/galvanostat Autolab PGSTAT204 (Metrohm Autolab, Utrecht, The Netherlands) within the voltage range of −1.2 to 1.2 V. The ^{1}H-NMR spectrum was recorded using the Avance 600 MHz spectrometer (Bruker, Billerica, MA, USA) and referenced to the solvent signals.

Agarose gel retardation

Sodium alginate and the obtained GNS/Alg-NGs samples synthesized at different temperatures (5 mg mL^{-1}; 15 μL) were separately mixed with 10% glycerol, then analyzed by gel electrophoresis in 1.0% agarose gel and 40 mM Tris-acetate buffer/1 mM ethylenediaminetetraacetic acid (TAE buffer, pH 8.0) at 100 V for 30 min. The gel was externally stained by the positively charged methylene blue (0.1 mg mL^{-1}, 150 mL) after electrophoresis and destained in TAE buffer (pH 8.0) for 30 min.

Determination of the dissociation constant of thrombin with GNS/Alg-NGs-270

The dissociation constant (K_d) for the interaction of GNS/Alg-NGs-270 with thrombin was determined to understand the anticoagulant properties of the GNS/Alg-NGs-270 against thrombin activity. Briefly, aliquots of GNS/Alg-NGs-270 solution (0.1 mg mL^{-1}) with different concentrations of thrombin (0–800 pM) were added to PBS containing BSA (100 μM, and incubated for 30 min to allow the interaction between thrombin and GNS/Alg-NGs-270. To further separate the thrombin-GNS/Alg-NGs-270 and free thrombin, the mixture (40 μL) was added into a size exclusion chromatography (SEC) column (Sephacryl S-200 HR, GE Healthcare, Chicago, IL, USA) with a bed volume of 1.0 mL. The sample solution of the fraction was eluted 7 times with 200 μL of PBS solution. The fractions 4–7 with free thrombin were collected, estimated to fibrinogen-modified gold nanoparticles (Au NPs; 56 nm) solution, and then the K_d value was calculated according to a previously published method.[1] The saturation binding data were processed using the Scatchard equation below to examine the binding properties.

$$N_{Thrombin}/[\text{Free-Thrombin}] = N_{max}/K_d - N_{Thrombin}/K_d$$

where, $N_{Thrombin}$ is the number of thrombin molecules bound to GNS/Alg-NGs-270 at equilibrium, [Free-Thrombin] is the unbound thrombin concentration at equilibrium, N_{max} is the apparent maximal number of binding sites, and K_d is the dissociation constant. The value $1/K_d$ was obtained from the slope of the plot of $N_{Thrombin}/[\text{Free-Thrombin}]$ against $N_{Thrombin}$, respectively.

The coagulation factor activity test

The activity of coagulation factors (II, V, VII, X, XI, XII) was determined by a Sysmex CS-2500 System (Siemens Healthineers, Erlangen, Germany). The GNS/Alg-NGs-270 (2.0 mg

mL^{-1}; 100 μL) and human plasma (900 μL) were mixed for analysis. Except for the measured clotting factor, the remaining clotting factors were added in excess, and then the activation agents were added (20 μL) directly before analysis. The clotting time of the various factors was determined by the detection of transmitted light (absorbance) at 660 nm. The raw data was then inputted into a mathematical algorithm from the instrument to determine the solidification time. All assays were performed according to the manufacturer's instructions with the assistance of the National Taiwan University Hospital. All tests were performed in triplicate.

DPPH radical scavenging activity

The radical scavenging activity of GNS/Alg-NGs was measured according to the method of Blois.[2] Briefly, various concentrations of untreated sodium alginate or GNS/Alg-NGs (1−100 μg mL^{-1}) were added to a DPPH solution (50 μM, diluted in EtOH) in a mixing solution of 50% PBS and 50% EtOH, and then reacted for 30 min at room temperature. The decrease in the solution absorbance was measured at 525 nm. The percentage DPPH radical scavenging activity was calculated using the following formula:

DPPH radical scavenging activity (%) = $[(A_0-A_1)/A_0 \times 100]$,

where A_0 and A_1 are the absorbance values in the absence and presence of alginate or GNS/Alg-NGs, respectively.

Folin-Ciocalteu assays

The total phenolic content of the GNS/Alg-NGs was measured by using the Folin-Ciocalteu reagent. Briefly, 40 μL of GNS/Alg-NGs were mixed with Folin−Ciocalteu reagent (240 μL) for 0.5 h, and 120 μL of sodium carbonate (7.5%, w/v) was added after 0.5 h. The absorbance was then measured at 760 nm after incubation at 25 ℃ for an additional 0.5 h. Results were expressed as standard quercetin equivalents (μg of quercetin / μg of GNS/Alg-NGs). The Folin-Ciocalteu assay is based on the transfer of electrons from phenolic compounds in basic solution to phosphomolybdic/phosphotungstic acid to form blue complexes $[(PMoW_{11}O_{40})^{4-}]$, which are quantitated by monitoring the absorbance at 760 nm. However, the Folin-Ciocalteu assay is not only sensitive to phenolic compounds but also many reducing agents.[3] Thus, this assay could be employed to measure the total reducing capacity of the sample.

Prothrombin time and activated partial thromboplastin time assays

The anticoagulation properties of GNS/Alg-NGs were further evaluated by determining the prothrombin time (PT) and activated partial thromboplastin time (aPTT) delay in human plasma samples. For the PT assay, sodium alginate or GNS/Alg-NGs (0.5 mg mL^{-1}, 100 μL) were separately incubated with 200 μL of human plasma at 37 ℃ for 3 min, followed by the addition of 200 μL of PT reagent to initiate the extrinsic clotting cascade. Coagulation was monitored by measuring the intensity of scattered light until the signals reached saturation. For the aPTT determinations, 160 μL of human plasma was incubated with 240 μL (0.5 mg mL^{-1}) of each inhibitor (sodium alginate or GNS/Alg-NGs) at 37 ℃ for 5 min. Then, 100 μL of aPTT reagent was added, followed by incubation for 3 min. To initiate the intrinsic clotting cascade, 100 μL of prewarmed $CaCl_2$ solution was added. The aPTT was noted as the time at which the intensity of the scattered light reached a plateau. To calculate the PT and aPTT, the end time was chosen as the point at which the scattered light signal intensity was halfway between the lowest and maximal values. Three repeated measurements were obtained for each set of experiments using a single batch of plasma.

Thromboelastography

We employed thromboelastography (TEG; thromboelastograph analyzer, Haemoscope Corporation, Niles, IL, USA) to further evaluate the anticoagulation efficiency of sodium alginate, GNS/Alg-NGs-270, and the well-known sulfated polysaccharide with prominent anticoagulant activity, fucoidan, in whole blood clots through the viscoelastic development of thrombin-mediated fibrin polymerization and platelet activation. Blood samples were collected from a healthy volunteer (24 years old) into tubes containing sodium citrate (3.2%). Blood collection at Mackay Memorial Hospital (MMH; Taipei, Taiwan) was performed in compliance with the relevant laws. Plain disposable plastic TEG cups (Haemonetics) were equilibrated at 37 ℃ before the experiments. On the thrombin-activated TEG assay, sodium alginate, GNS/Alg-NGs-270, fucoidan, or other commercial drugs (0.1–0.5 mg mL^{-1}, 52 μL) in physiological buffer was mixed with 288 μL of whole blood and incubated in a TEG cup at 37 ℃ for 10 min. Then, the thrombin solution (15 nM, 20 μL) was added to the TEG cup to initiate whole-blood coagulation and then monitored for 30 min. Likewise, the same

concentration of samples was mixed with the same amount of whole blood (288 μL) and kaolin solution (14 μL), then incubated in a TEG cup at 37 ℃ for 10 min on the kaolin-activated TEG assay. Then, the calcium chloride solution ($CaCl_2$, 14 μL) was added to the TEG cup to initiate whole-blood coagulation and then monitored for 30 min. The clot formation at 37 ℃ was measured with the thromboelastography analyzer until a stable clot had formed or one hour had passed. The degree of motion was set to 4.75° around a fixed plastic pin to measure the clot reaction time (R) (minutes), clot kinetics (K, time from clot formation to the time that the amplitude reaches 20 mm) (minutes), α-angle (α, formed by the slope from the R-value to the K-value representing the acceleration of fibrin build-up and cross-linking) (degree) and maximum amplitude (MA, amplitude measured at the widest point of TEG tracing).[4] The lag time, α angle, and maximum amplitude were automatically calculated by the TEG® Analytical software (TAS) version 4.2.3 (Haemonetics).

In vitro cytotoxicity assay

We studied the *in vitro* cytotoxicity of GNS/Alg-NGs-270 in HUVEC (human umbilical vein endothelial cell line), HEK-293T (human embryonic kidney 293 cell line), RD (human rhabdomyosarcoma cell line), HepG2 (human liver cancer cell line), and A549 (human lung adenocarcinoma epithelial cell line) cells to establish their bio-cytotoxicity. All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HUVEC and A549 cell lines were cultured in M199 and F-12K medium, respectively, and HEK-293T, RD, and HepG2 cell lines were cultured in the DMEM medium. The medium was supplemented with fetal bovine serum (FBS, 10%), antibiotic-antimycotic (1%), L-glutamine (2.0 mM), and nonessential amino acids (NEAA, 1%) in 5% CO_2 at 37 ℃. The cell number was determined by the trypan blue exclusion assay (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), and the cell viability was determined using the PrestoBlue assay. Briefly, approximately 1.0×10^4 cells per well of all cell lines were separately incubated with the respective culture medium for 12 h at 37 ℃ containing 5% CO_2 in 96-well plates. Then, the culture medium in each well was replaced with 100 μL of medium containing GNS/Alg-NGs-270 (0.1–1.0 mg mL^{-1}), followed by incubation for 72 h. The cells were carefully rinsed thrice with PBS and treated with the PrestoBlue reagent (ten-fold dilution, 100 μL per well) for 4 h. Then, the fluorescence intensities (I_{F590}) were measured at an excitation/emission

wavelength of 540/590 nm (Synergy 4 Multi-Mode; BioTek Instruments). Because fluorescence intensity is directly correlated with cell quantity, the cell viability in the control set (media without GNS/Alg-NGs-270) was assumed to be 100%. The LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen) was further used to examine live and dead cells. The HEK-293T cells were maintained in a cell culture chamber dissolved in DMEM medium at 37 ℃ humidified atmosphere containing 95% air and 5% CO_2. HEK-293T cell lines were cultured in 24-well plates for 72 h (*ca.* 1.0×10^5 cells/well). Subsequently, the culture solution was replaced with a culture solution containing GNS/Alg-NGs-270 (0.1–1.0 mg mL^{-1}) and cultured for 72 h. The HEK-293T cell cultures, after washing three times with PBS, were stained with a working solution consisting of PBS (1.0 mL), EthD-1 (2.0 μL), and calcein-AM (0.5 μL) and viewed under a fluorescence microscope (Axiovert 200 M, Carl Zeiss, Oberkochen, Germany). Calcein AM is a non-fluorescent dye that easily permeates live mammalian cells with an intact cellular membrane. The hydrolysis of Calcein AM by intracellular esterases produces Calcein, which is well-retained in the cell cytoplasm. Calcein exhibits strong green fluorescence at 520 nm upon excitation at 480–500 nm. EthD-1 is excluded by the intact plasma membrane of live cells. EthD-1 enters cells with damaged cellular membranes and undergoes an enhancement of fluorescence (*ca.* 40-fold) upon binding to nucleic acids, thereby producing a strong red fluorescence (*ca.* 635 nm) in dead cells upon excitation at 480–500 nm.

Hemolysis assays

The human red blood cells (RBCs) for the hemolysis assays were donated from MMH (Taipei, Taiwan). A hemolysis assay to test the GNS/Alg-NGs-270 was performed following a previous report.[5] Blood samples were collected from a healthy volunteer (male, 25 years old) into tubes containing ethylenediaminetetraacetic acid (EDTA) and immediately (within 30 min of the collection) centrifuged (RCF 3,000 *g*, 10 min, 4 ℃) to remove the serum. The RBCs were diluted with sterile isotonic physiological buffer to obtain an RBC stock suspension (*ca.* 4.0 vol% blood cells). For the analysis, 200 μL of the RBC stock suspension was incubated with aliquots of GNS/Alg-NGs-270 dispersions (0.1–1.0 mg mL^{-1}) in 1.5 mL vials at 37 ℃ for 1 h. Then, the aliquots were centrifuged at an RCF of 1,000 *g* for 10 min. Hemolysis was measured based on the absorption of hemoglobin at 576 nm (OD_{576}) in the supernatant (150

μL). For the 0% hemolysis reference ($OD_{576\ blank}$), a sterile isotonic physiological buffer was used. The positive control expressing 100% hemolysis was prepared by adding ultrapure water to an RBC suspension ($OD_{576\ ultrapure\ water}$). The hemolysis activity was calculated as follows: Hemolysis (%) = $[(OD_{576\ GNS/Alg\text{-}NGs\text{-}270} - OD_{576\ blank})/(OD_{576\ ultrapure\ water} - OD_{576\ blank})]\times 100\%$.

In vivo biocompatibility evaluation

The *in vivo* toxicity of GNS/Alg-NGs-270 was investigated by employing male Sprague-Dawley rats (200–250 g). All protocols involving animal handling were performed within the Guidelines for Care and Use of Laboratory Animals of National Taiwan Ocean University and approved by the Institutional Animal Care and Use Committee of the National Laboratory Animal Center, Taipei, Taiwan (IACUC Approval No. 105021). The mice were anesthetized with a mixture containing Zoletil and xylazine. The GNS/Alg-NGs-270 (1.0 mg kg^{-1}) was intravenously administered to healthy rats, and their body weight was monitored over 14 days. The blood samples were collected from the vein of PBS and GNS/Alg-NGs-270 treated rats after day 15 and levels of alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (T-BIL), gamma-glutamyl transferase (GGT), and blood urea nitrogen (BUN) were estimated using HITACHI 7180 medium-sized biochemistry automatic analyzer. The histological analysis of the rat was sacrificed on day 15 and carried out by staining the major organs (e.g., heart, liver, spleen, lung, kidney) with hematoxylin and eosin stain (H&E staining).

Statistics

Results were expressed as mean ± standard deviation. Comparative studies of means were carried out using the Student's *t*-test. Significance was accepted with $p < 0.05$.

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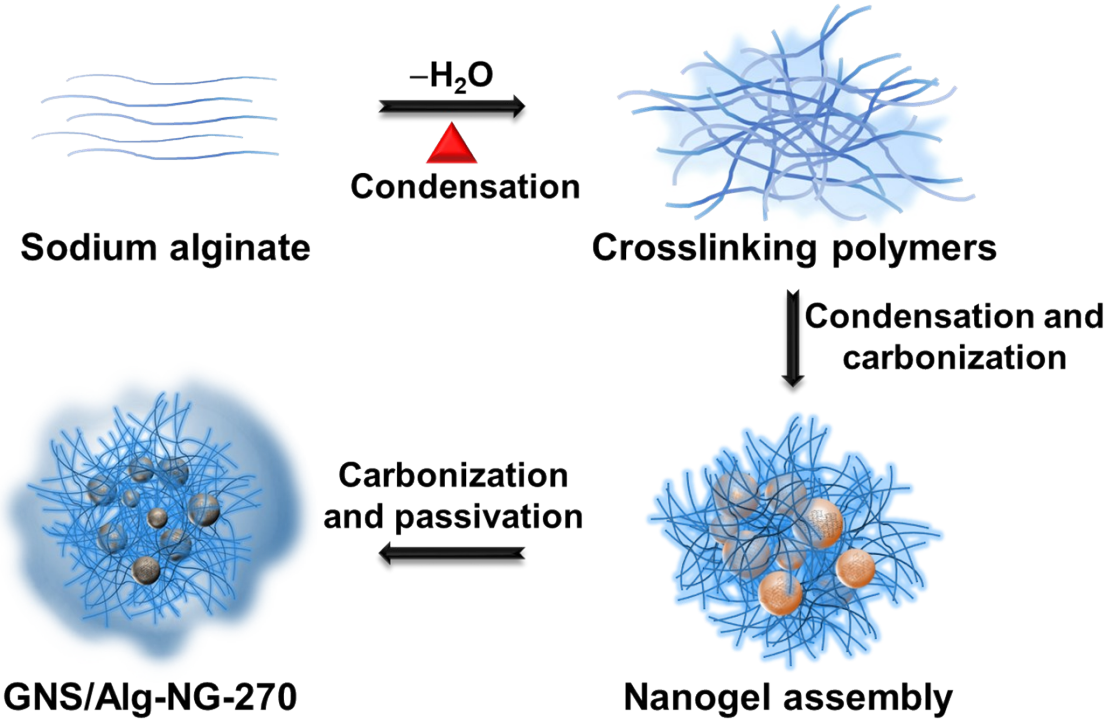
Table S1 Product yield, zeta potential, hydrodynamic diameter, fluorescence quantum yield (QY), and elemental compositions of the as-prepared products from sodium alginate without treatment and after dry heating.

		Product yield (%)	Zeta potential (mV; *n* = 5)	Hydrodynamic diameter (nm; *n* = 5)	Fluorescence QY (%)[a]	Elemental compositions (wt%)[b]		
						C	H	O
Untreated sodium alginate		-	–86.2 ± 2.1	474.6 ± 155.7	<0.01	29.98	5.02	55.33
Sodium alginate	**150** ℃	95.2	–45.8 ± 1.3	410.2 ± 77.9	<0.01	29.38	5.20	55.63
	180 ℃	85.3	–41.8 ± 2.4	327.4 ± 65.3	14.30	30.74	5.00	54.61
	210 ℃	60.9	–40.5 ± 6.9	153.1 ± 38.4	8.30	36.71	4.63	46.16
	240 ℃	48.3	–34.2 ± 4.2	78.5 ± 25.7	4.48	34.83	4.88	45.48
	270 ℃	46.8	–31.7 ± 5.1	52.0 ± 11.3	3.22	33.53	4.81	44.57
	300 ℃	36.1	–30.4 ± 0.8	198.2 ± 49.9	2.09	35.19	3.92	44.00

[a] compared to quinine sulfate (QY: 54% in 0.1 M H_2SO_4).

[b] determined by elemental analysis.



Scheme S1 The proposed mechanism of the formation of GNS/Alg-NGs-270.

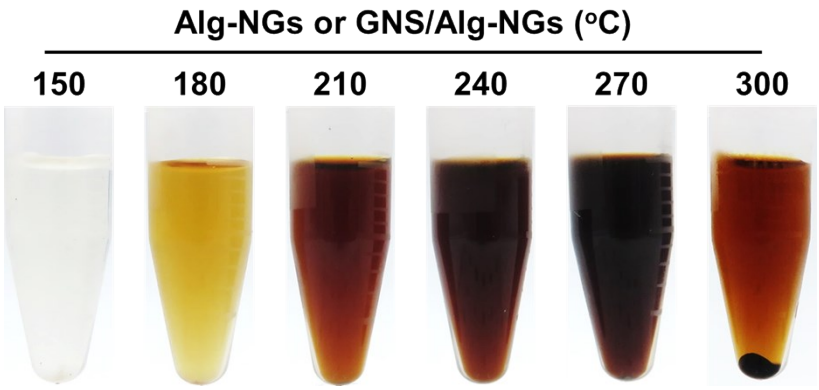


Fig. S1 Photographs of the dispersions of Alg-NGs-150, Alg-NGs-180, GNS/Alg-NGs-210, GNS/Alg-NGs-240, GNS/Alg-NGs-270, and GNS/Alg-NGs-300 suspended in deionized water.

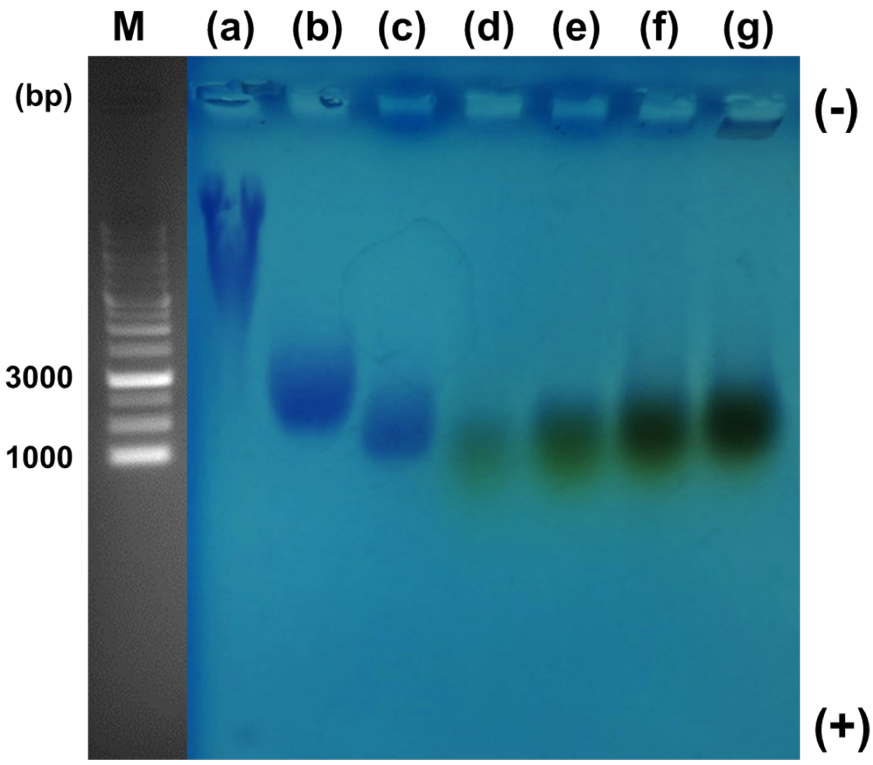


Fig. S2 Agarose gel electrophoresis to assess the charge and size distribution of alginate, Alg-NGs, and GNS/Alg-NGs. (a) Sodium alginate, (b) Alg-NGs-150, (c) Alg-NGs-180, (d) GNS/Alg-NGs-210, (e) GNS/Alg-NGs-240, (f) GNS/Alg-NGs-270, and (g) GNS/Alg-NGs-300. The concentration was 5 mg mL^{-1}. "M" denotes the GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific) used to collate the size distribution of the as-prepared products. Gel separation was performed in 1.0% agarose under a constant electric field (100 V cm^{-1}) for 30 min. TAE Buffer, 40 mM Tris-acetate/1 mM EDTA, pH 8.0. Methylene blue (MB) was used as a staining agent.

Sodium alginate shows broadband, probably due to the polymers with a wide range of molecular mass (**Fig. S2(a)**). Alg-NGs-150 and Alg-NGs-180 (**Fig. S2(b,c)** show a narrower distribution and lower band location than that of untreated sodium alginate, which indicates the formation of condensed polymers or supramolecular structures, probably through dehydration and condensation reactions. GNS/Alg-NGs-210, GNS/Alg-NGs-240, GNS/Alg-NGs-270, and GNS/Alg-NGs-300 (**Fig. S2(d–g)**, show their yellowish or brown color in an agarose gel under visible light. Their sharp band and lower band location compared to untreated sodium alginate and Alg-NGs suggest that GNS/Alg-NGs have a relatively narrow size distribution and possess a negative charge even after partial carbonization.

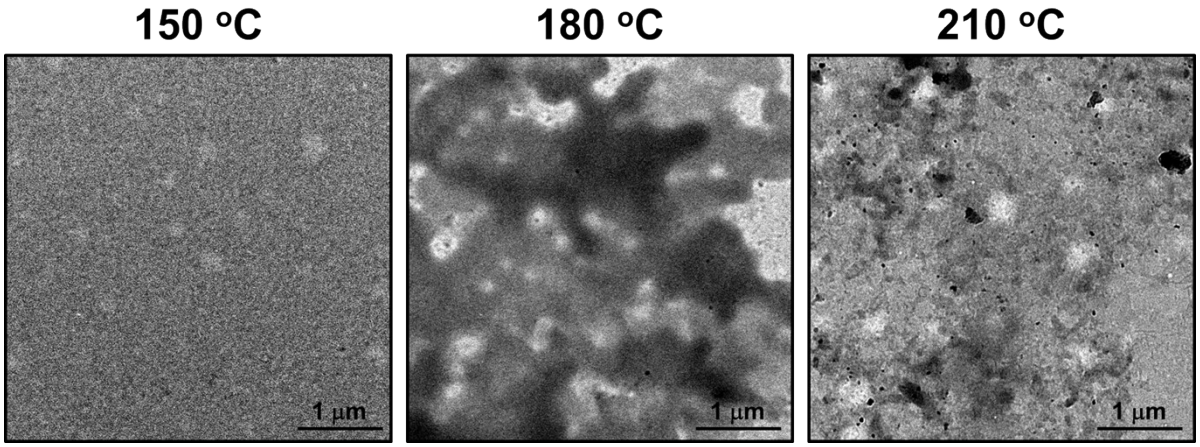


Fig. S3 Low magnification TEM images of the Alg-NGs prepared at 150 and 180 ℃ and GNS/Alg-NGs prepared at 210 ℃.

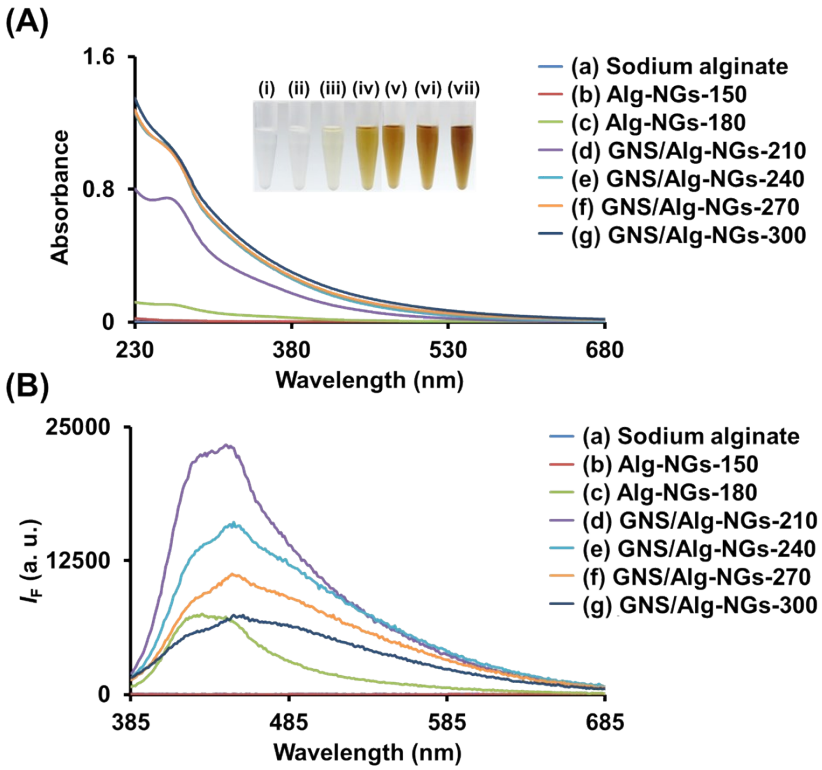


Fig. S4 (A) UV-vis absorption and (B) fluorescence spectra of alginate, Alg-NGs, and GNS/Alg-NGs. (a) sodium alginate, (b) Alg-NGs-150, (c) Alg-NGs-180, (d) GNS/Alg-NGs-210, (e) GNS/Alg-NGs-240, (f) GNS/Alg-NGs-270, and (g) GNS/Alg-NGs-300. The concentration of sodium alginate, Alg-NGs, or GNS/Alg-NGs was 1.0 mg mL^{-1} in 5 mM sodium phosphate buffer (pH 7.4) for spectroscopic measurements. Inset to (A) is the photograph of the corresponding solutions under visible light. Fluorescence spectra of all samples were recorded at an excitation wavelength of 365 nm. The fluorescence (I_F) intensities are plotted in arbitrary units (a. u.).

The fluorescence quantum yields (QYs) of the sodium alginate is <0.01% (**Table S1**), and for the Alg-NGs and GNS/Alg-NGs synthesized at 150–300 ℃ it was determined to be 2.09–14.30% by comparison with quinine (QY = 54% in 0.1 M H_2SO_4) as the reference. The excitation wavelength-dependent fluorescence emission (**Fig. S5**) of Alg-NGs and GNS/Alg-NGs mainly reflects the self-trapping of multiexcitons in aromatic networks and/or different surface emissive sites.[6,7] Although the particle morphology, size, and oxygen content in the GNS/Alg-NGs are quite different, the emission profiles of these GNS/Alg-NGs are similar. Therefore, these results suggest that the fluorescence of the GNS/Alg-NGs is mainly attributed to the as-formed graphene-like nanosheets embedded in cross-linked polymers (nanogels).[8]

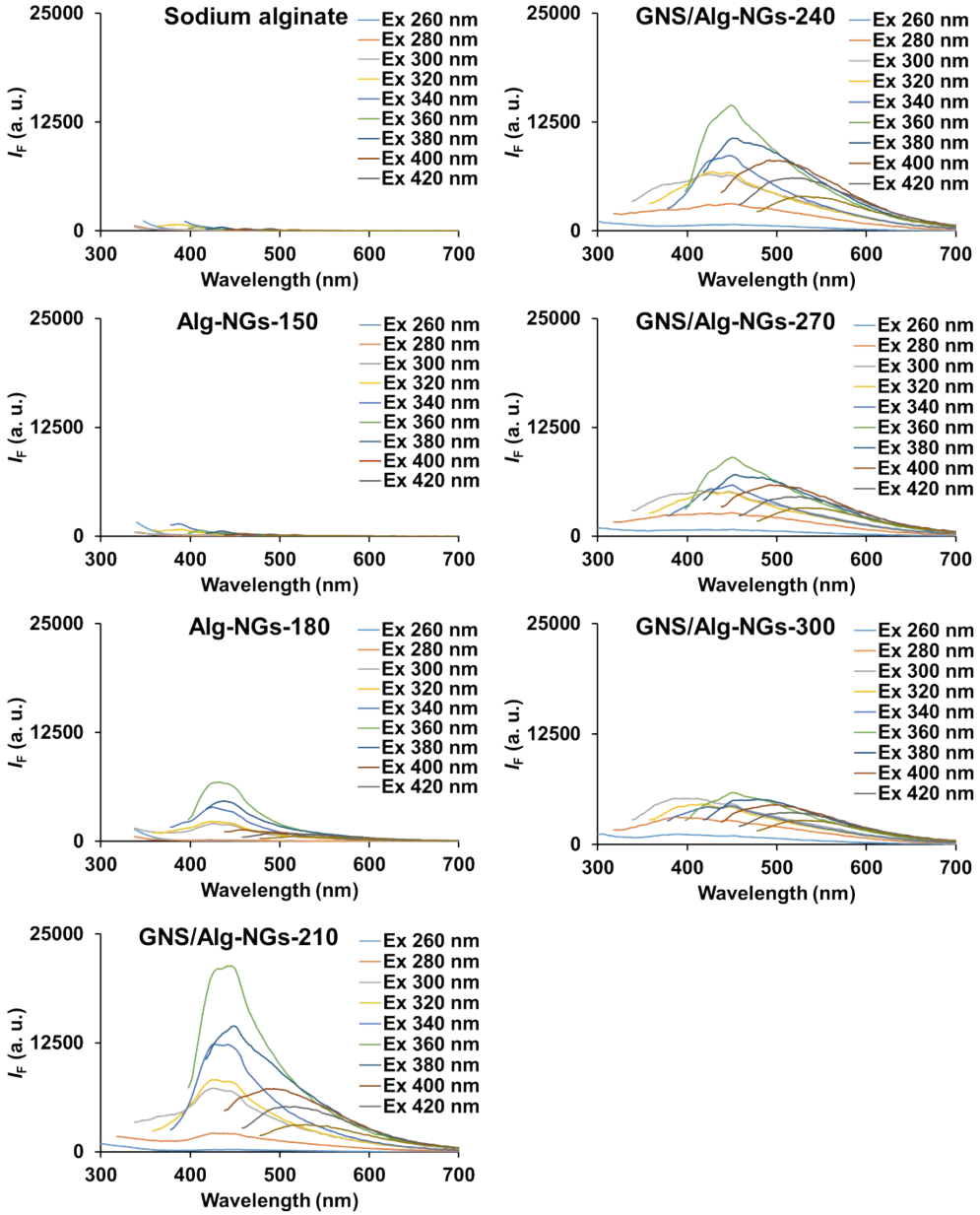


Fig. S5 Excitation-dependent emission spectra of sodium alginate, Alg-NGs, and GNS/Alg-NGs. Sodium alginate, Alg-NGs-150, Alg-NGs-180, GNS/Alg-NGs-210, GNS/Alg-NGs-240, GNS/Alg-NGs-270, and GNS/Alg-NGs-300 were at 0.1 mg mL^{-1} in 5 mM sodium phosphate buffer (pH 7.4) under different excitation wavelengths (from 260 to 440 nm; in 20 nm increments).

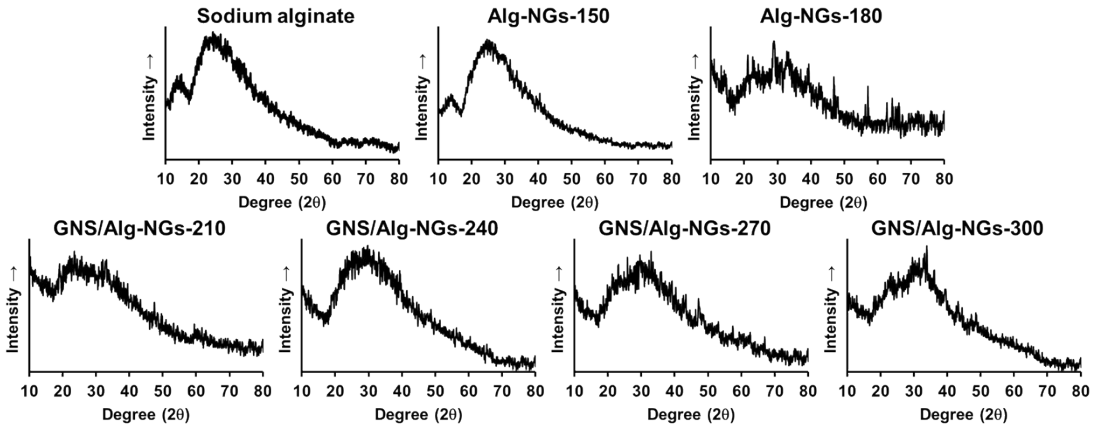


Fig. S6 XRD spectra of alginate, Alg-NGs, and GNS/Alg-NGs.

Sodium alginate shows a sharp band centered at *ca.* 13.5° (2θ), which reflects the (110) plane of crystalline polyguluronate (G form) due to the multiple interactions between the alginate chains *via* intermolecular hydrogen bonding, and a broad peak at 15°–35°, which could be ascribed to amorphous structures.[9] The diffraction peak at 13.5° gradually decreases and then disappears with the increase in synthesis temperature, indicating a significant change in the alignment of G form units in the nanomaterials. The lateral interaction among the alginate chains and G form molecular crystals play a vital role in the formation of Alg-NGs during the dry heating process. The XRD peak of the G-form crystal is also observed in the Alg-NGs-150, suggesting that the G form of alginate feature is preserved after heating at 150 ℃. Alg-NGs-180 possesses less G form characteristics and amorphous structures of alginate. The XRD diffraction pattern of the GNS/Alg-NGs-240, GNS/Alg-NGs-270, and GNS/Alg-NGs-300 exhibit a typical broad peak at *ca.* 26.5° (2θ) corresponding to graphene inter-planar spacing (002) of 0.33 nm for the facets of the graphene-like plane structure. These XRD results imply that some alginate structures are preserved in the obtained Alg-NGs.

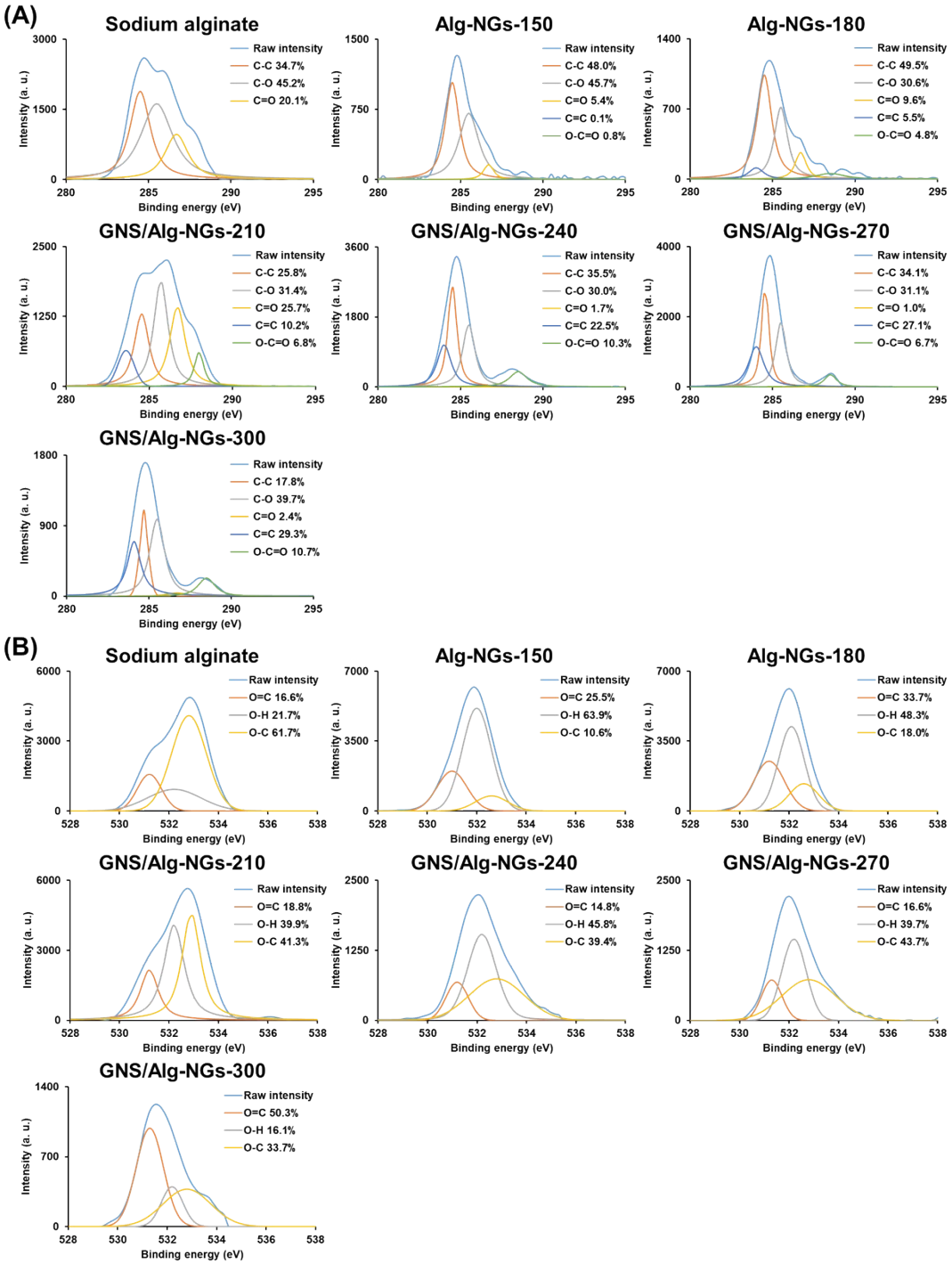


Fig. S7 (A) C1s and (B) O1s XPS spectra of alginate, Alg-NGs, and GNS/Alg-NGs.

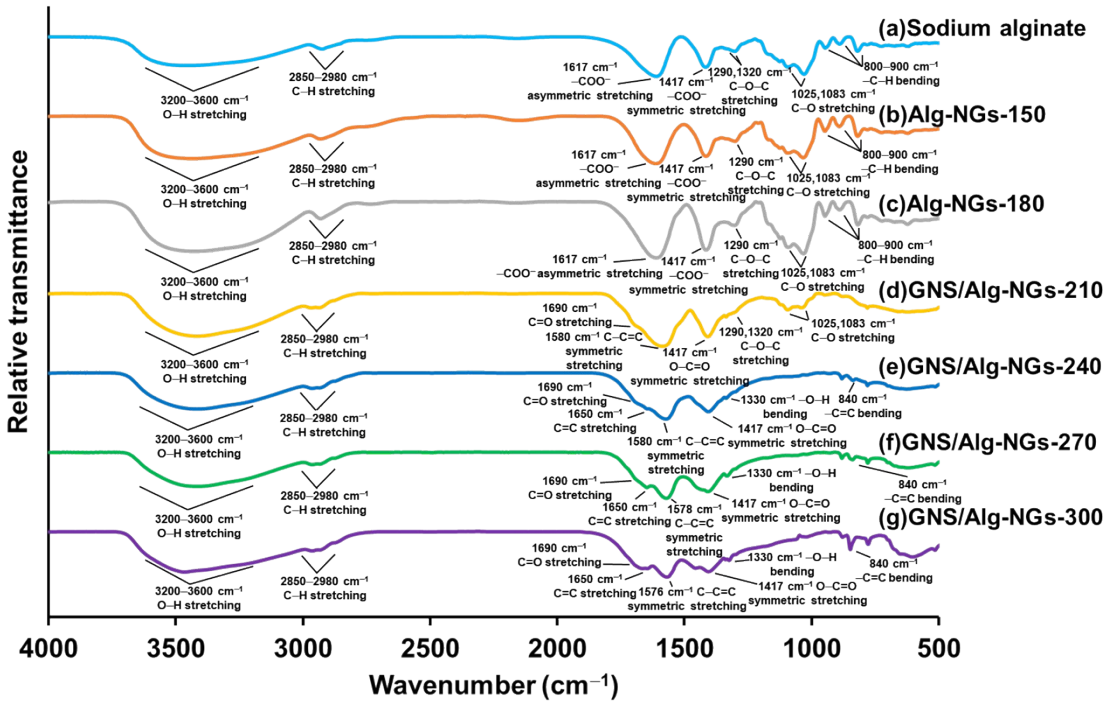


Fig. S8 FT-IR spectra of alginate, Alg-NGs, and GNS/Alg-NGs. (a) sodium alginate, (b) Alg-NGs-150, (c) Alg-NGs-180, (d) GNS/Alg-NGs-210, (e) GNS/Alg-NGs-240, (f) GNS/Alg-NGs-270, and (g) GNS/Alg-NGs-300.

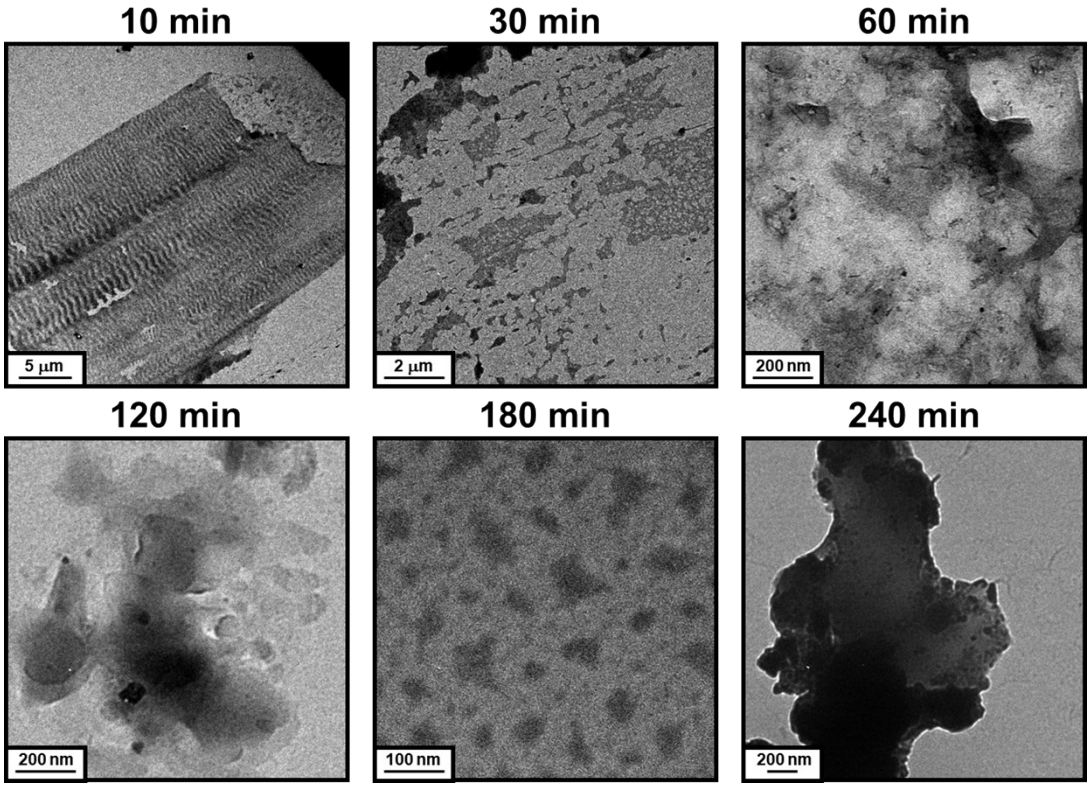


Fig. S9 TEM images of the time-course formation of GNS/Alg-NGs by heating sodium alginate at 270 ℃ for 10–240 min.

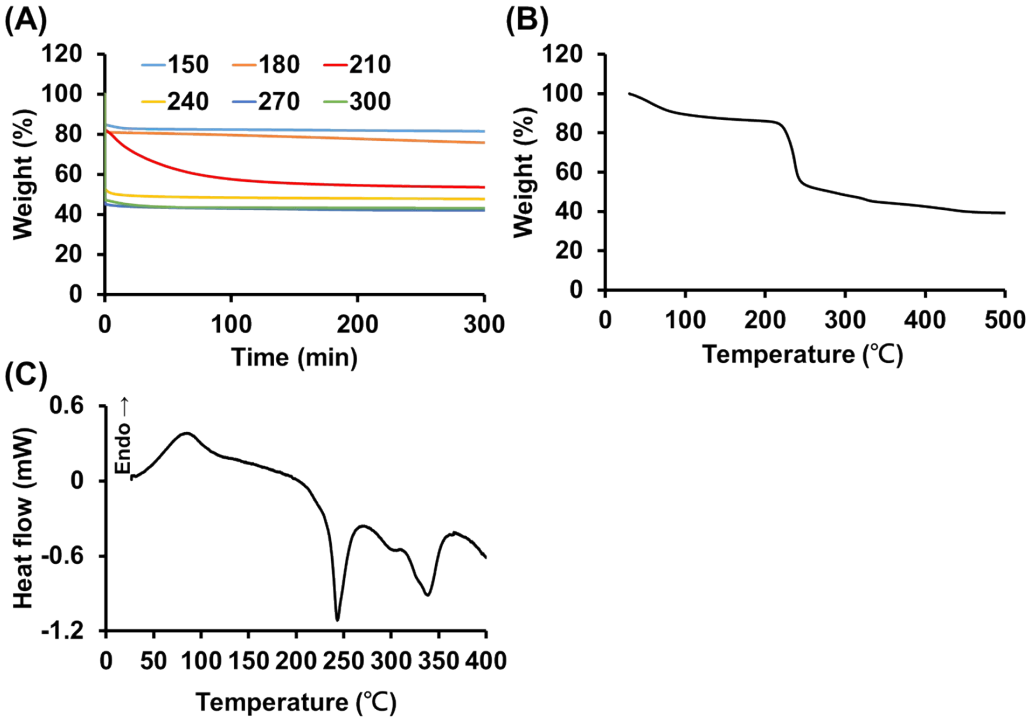


Fig. S10 Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) of sodium alginate. (A) Constant TGA data of sodium alginate at 150–300 ℃. (B) Temperature-dependent TGA and (C) DSC curve of sodium alginate. The heating rate in (B) and (C) were both 5 ℃ min^{-1}. Endo: endothermic.

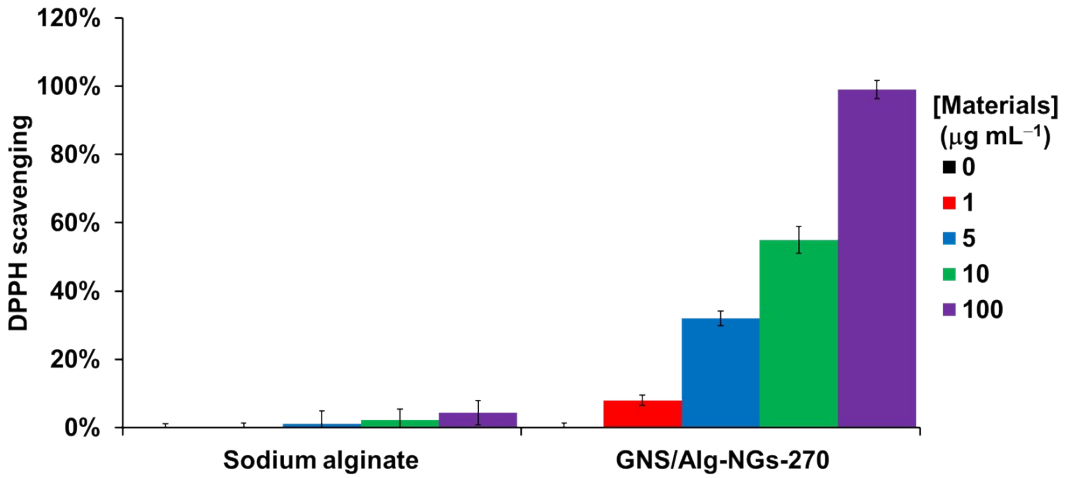


Fig. S11 DPPH scavenging activity of sodium alginate and GNS/Alg-NGs-270. Sodium alginate or GNS/Alg-NGs-270 was prepared in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 140 mM NaCl, and 2.70 mM KCl; adjusted to pH 7.4 using HCl) (pH 7.4)), then the absorbance of DPPH• radicals (50 μM) at 525 nm was recorded after the reaction for 0.5 h. Values are mean ± standard deviation (n = 3).

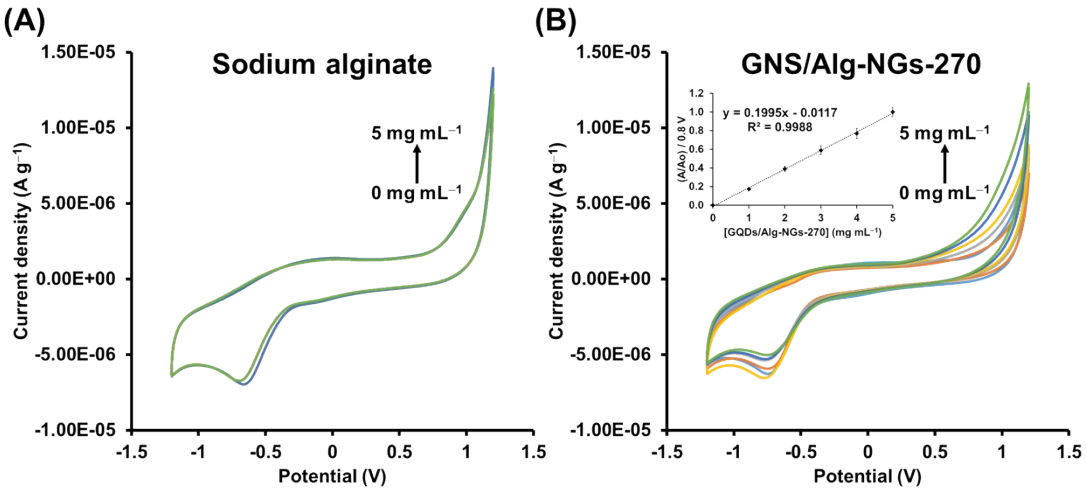


Fig. S12 Cyclic voltammograms of (A) sodium alginate and (B) GNS/Alg-NGs-270 in PBS buffer at various concentrations (0–5.0 mg mL^{-1}) on a glassy carbon electrode. The scan rate was 50 mV s^{-1}.

The oxidation peak corresponding to phenolic groups is not observed for sodium alginate. The oxidation peak at 0.8 V is observed for GNS/Alg-NGs-270, and the current density increases with the increased concentration (the inset to **Fig. S12B**).

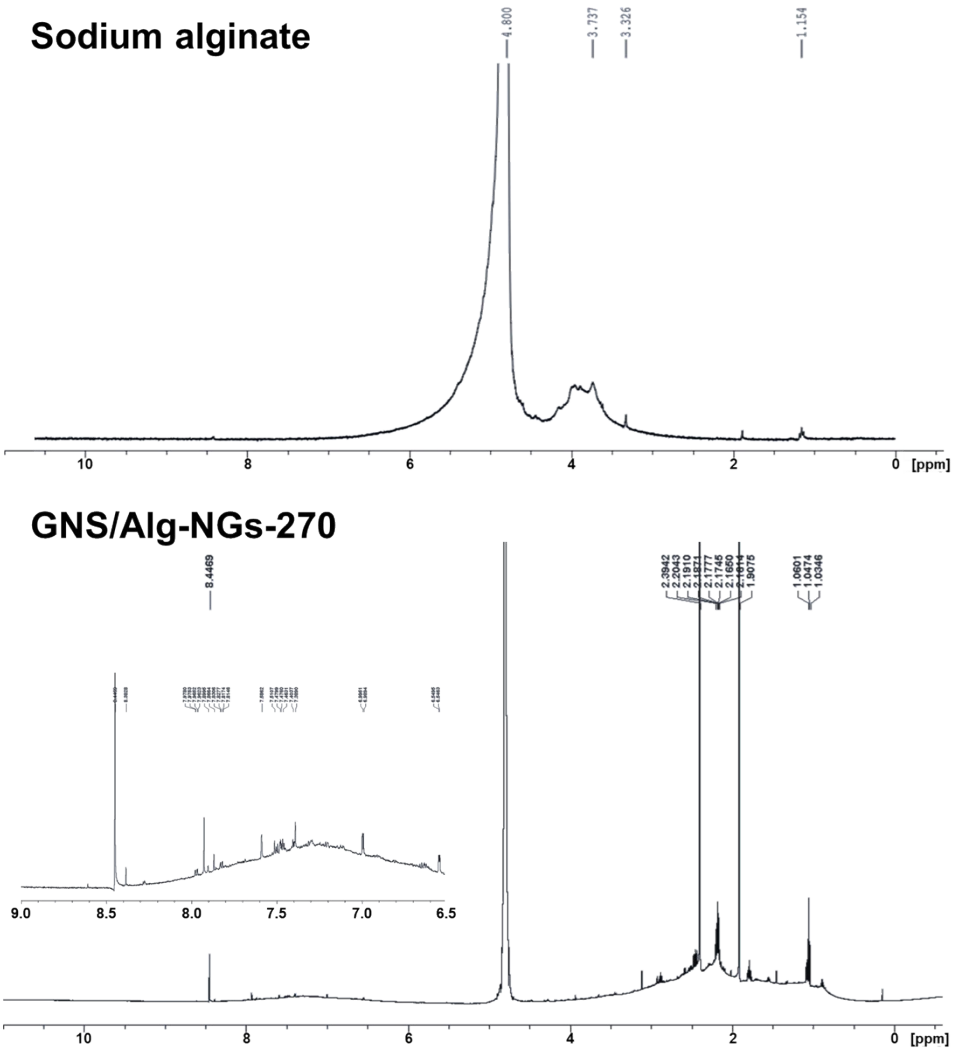


Fig. S13 The ^{1}H-NMR spectra of sodium alginate and GNS/Alg-NGs-270 at a concentration of 50 mg mL^{-1} in D_2O at 298 K. Inset in GNS/Alg-NGs-270 is the spectra from 6.5–9.0 ppm.

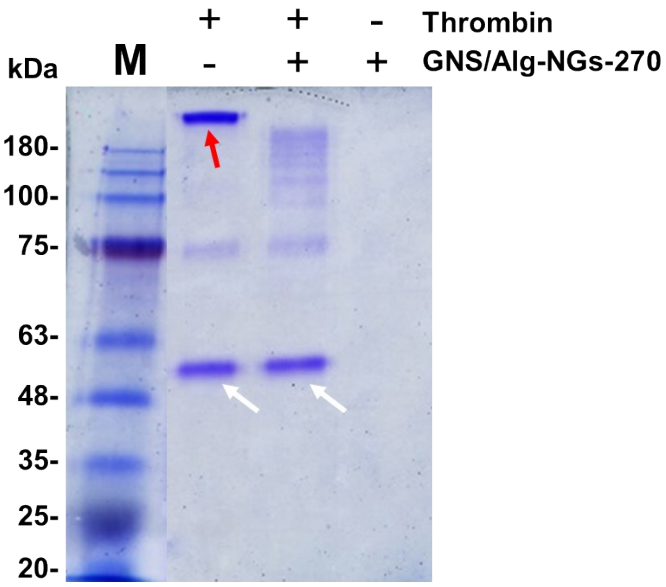


Fig. S14 Native polyacrylamide gel electrophoresis (native-PAGE) of thrombin and the mixture of GNS/Alg-NGs-270 and thrombin. The concentrations of thrombin and GNS/Alg-NGs-270 in PBS solution containing bovine serum albumin (BSA; 1300 μg mL^{-1}) were 130 μg mL^{-1} and 1.0 mg mL^{-1}, respectively. M: Bio-Helix™ Prestained Protein Ladder (PM007-0500) was used to collate the size distribution of the samples. The native PAGE (12%) was performed under a constant electric field (100 V cm^{-1}) for 80 min, and visualized by Coomassie Brilliant Blue R-250 staining. The white and red arrows show the BSA and thrombin, respectively.

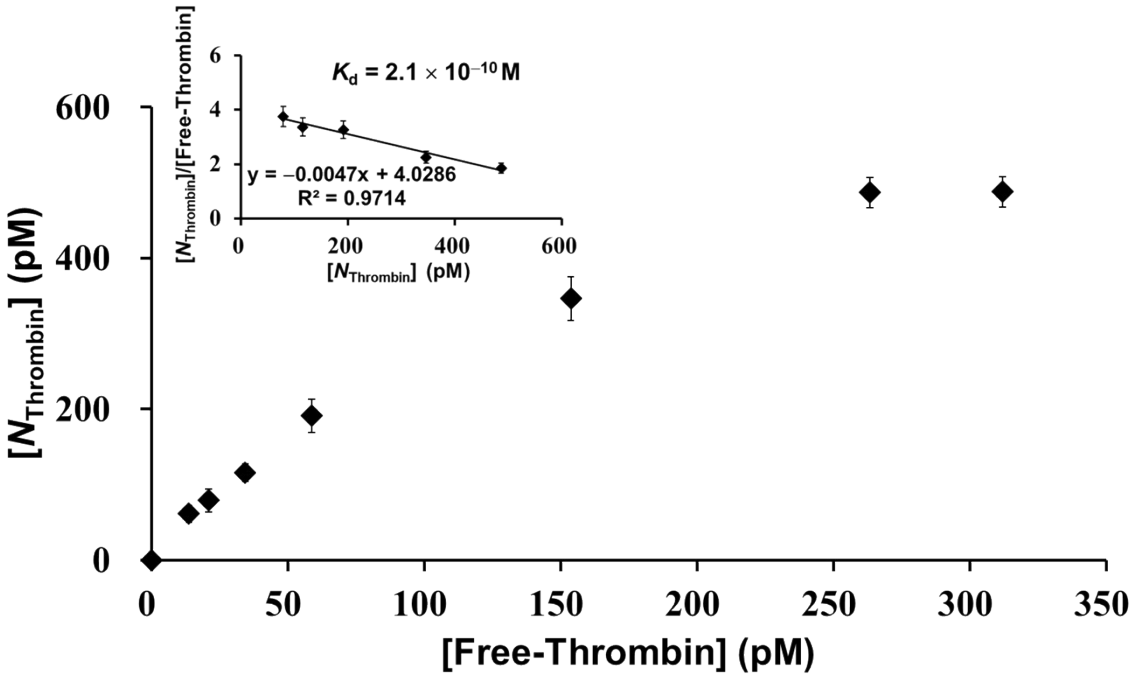


Fig. S15 The plot for calculation of the dissociation constants (K_d) of thrombin and GNS/Alg-NGs-270. [$N_{Thrombin}$] is the number of thrombin molecules bound to GNS/Alg-NGs-270 at equilibrium, and [Free-Thrombin] is the free thrombin concentration at equilibrium.

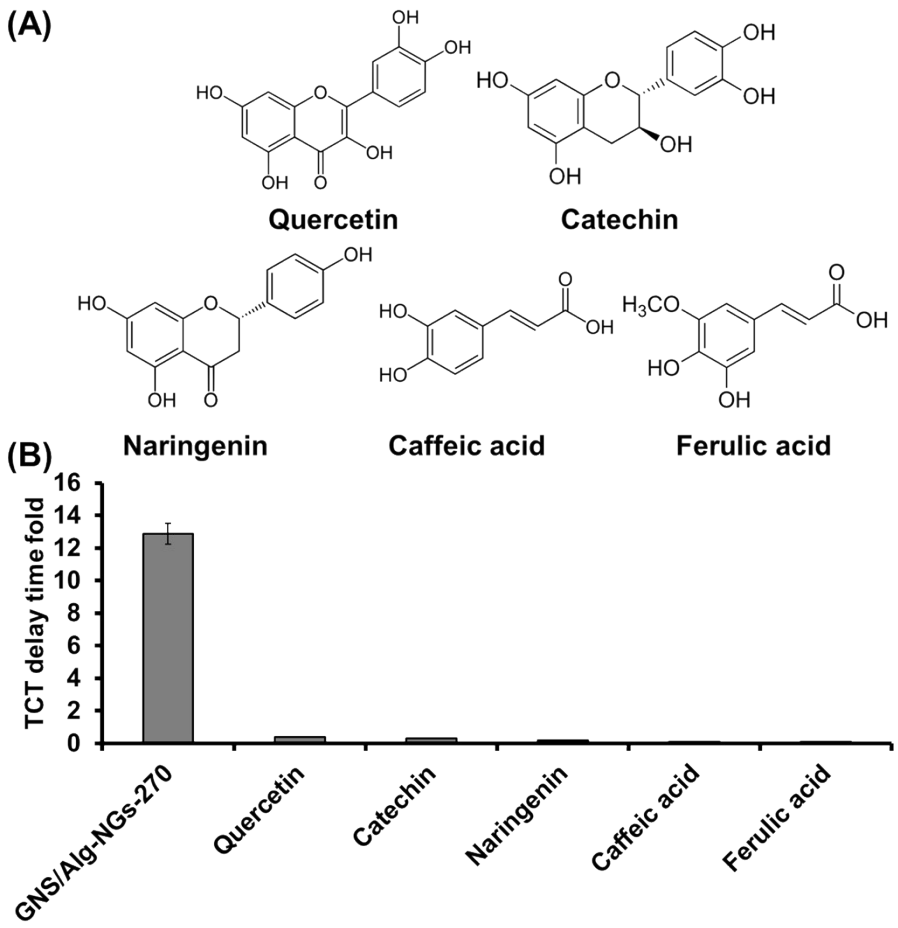


Fig. S16 Comparison of thrombin inhibitory activity of polyphenolic GNS/Alg-NGs-270 and natural (poly)phenolic compounds. (A) Molecular structure of quercetin, catechin, naringenin, caffeic acid, and ferulic acid. (B) TCT obtained from the light scattering intensities generated by the coagulation process in the presence of GNS/Alg-NGs-270, quercetin, catechin, naringenin, caffeic acid, and ferulic acid at a concentration of 0.5 mg mL^{-1}. Other conditions were the same as those described in **Fig. 2**.

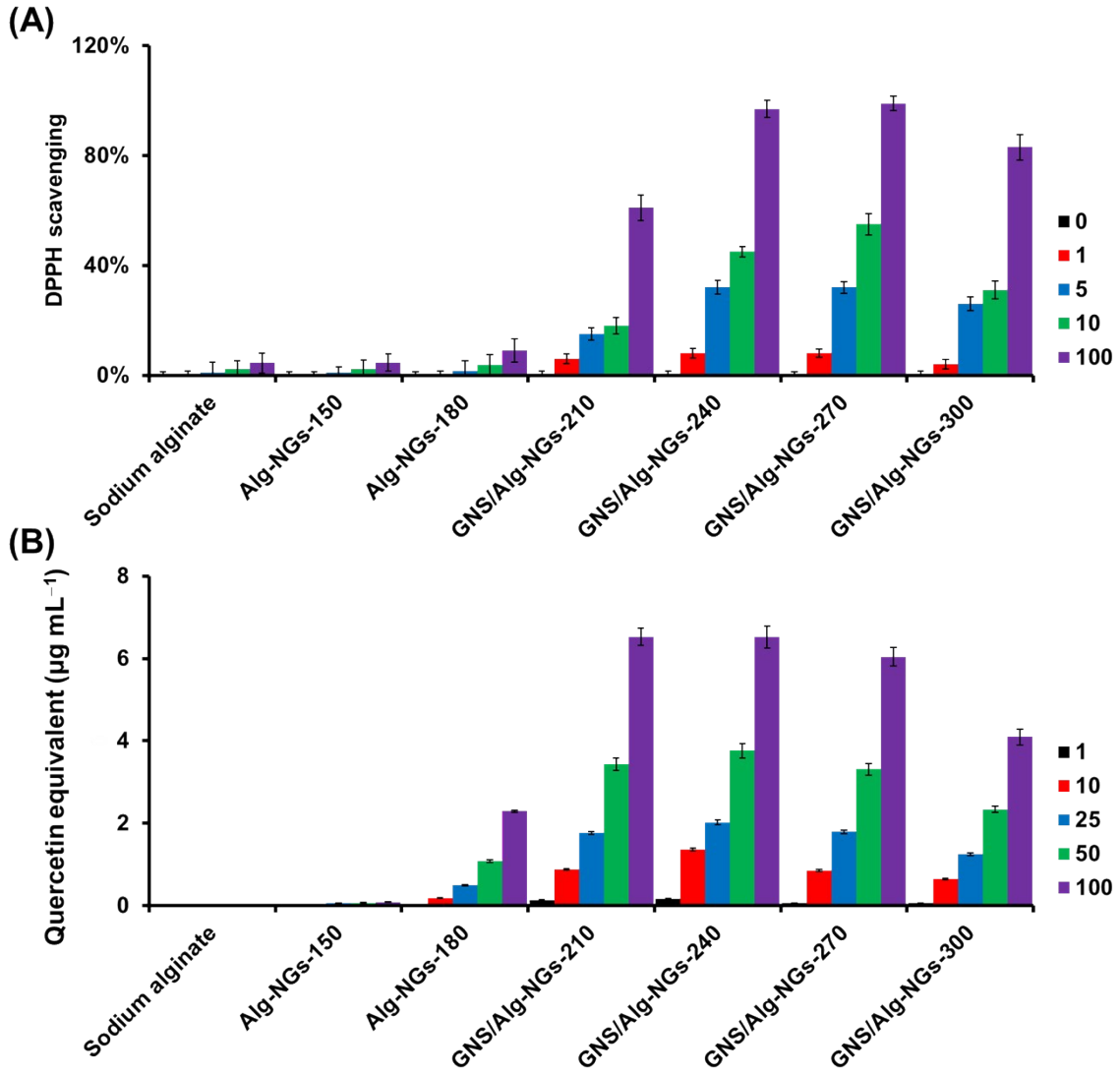


Fig. S17 Comparison of antioxidant activity of Alg-NGs and GNS/Alg-NGs. (A) DPPH scavenging and (B) Folin-Ciocalteu assays of sodium alginate, Alg-NGs-150, Alg-NGs-180, GNS/Alg-NGs-210, GNS/Alg-NGs-240, GNS/Alg-NGs-270, and GNS/Alg-NGs-300 at concentrations of 1–100 μg mL^{-1}. Values are mean ± standard deviation (n = 3). Other conditions were the same as those described in **Fig. S11**.

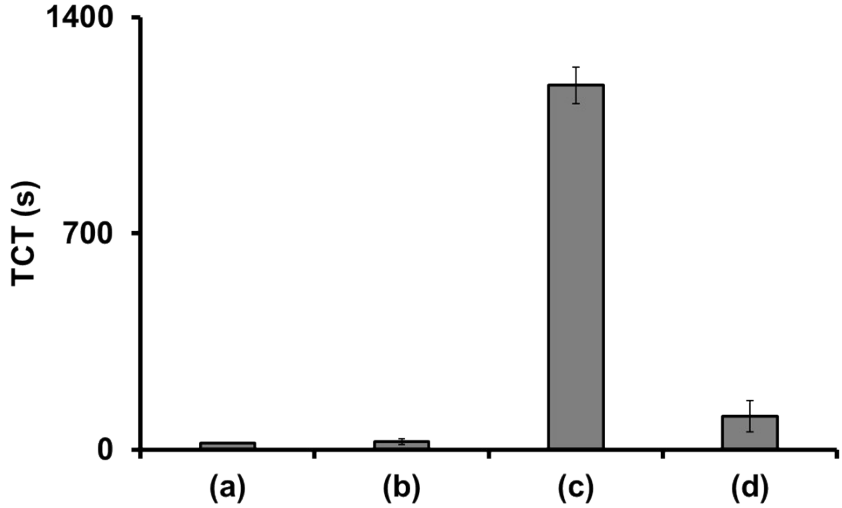


Fig. S18 The thrombin clotting time (TCT) assay of GNS/Alg-NGs-270 after treatment with H_2O_2. TCT obtained from the light scattering intensities generated by the coagulation process of (a) in the absence (as a control) and (b–d) in the presence of (b) H_2O_2 (50 mM), (c) GNS/Alg-NGs-270 (0.5 mg mL^{-1}), and (d) H_2O_2 (50 mM)-treated GNS/Alg-NGs-270 (0.5 mg mL^{-1}). The GNS/Alg-NGs-270 (0.5 mg mL^{-1}) was treated with H_2O_2 (50 mM) at room temperature for 2 h for further TCT assay. Error bars represent the standard deviation of three repeated measurements. Other conditions were the same as those described in **Fig. 2**.

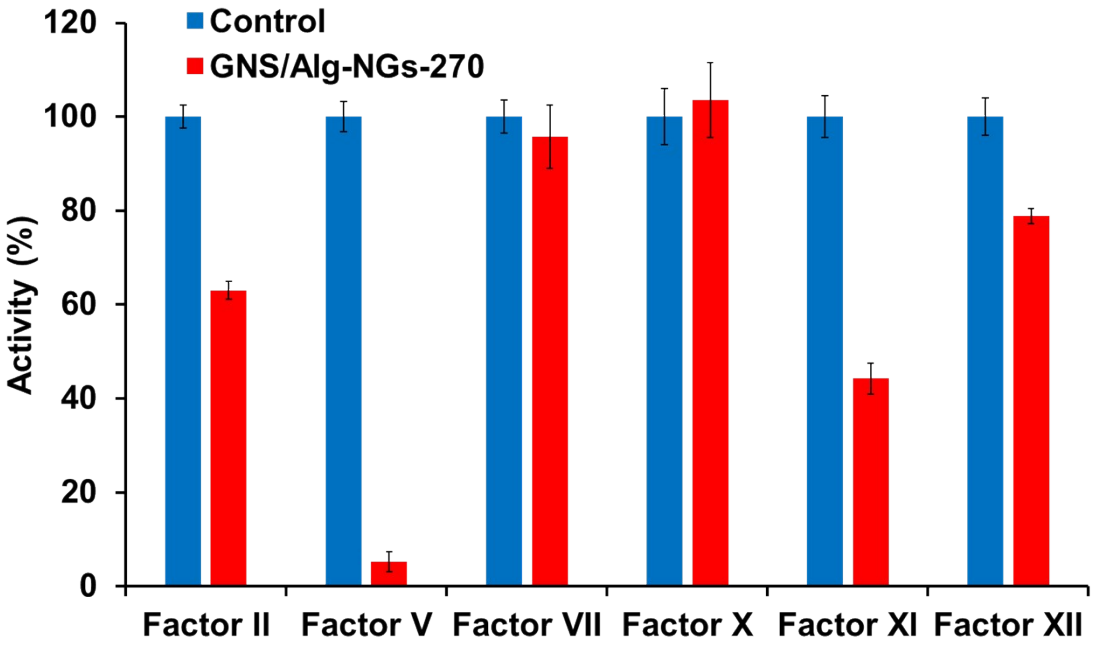
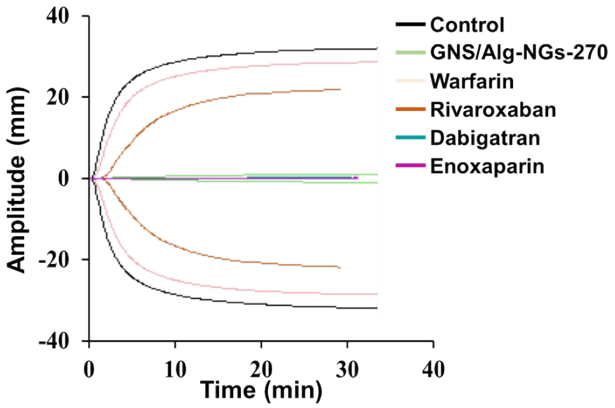


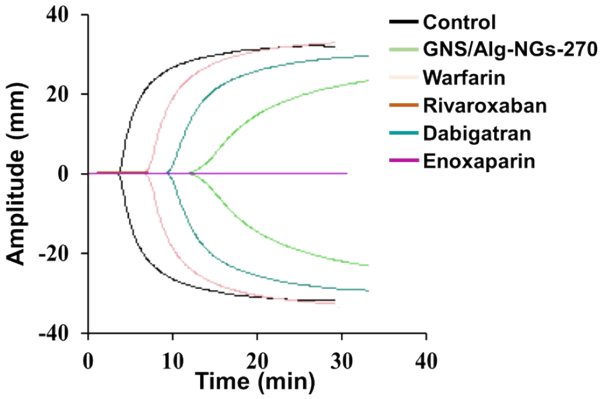
Fig. S19 The relative activity of selected coagulation factors (factor II, V, VII, X, XI, and XII) in the absence (control) and presence of GNS/Alg-NGs-270 (2.0 mg mL^{-1}) in human plasma. Error bars represent the standard deviation of three repeated measurements.

(A)



Inhibitor	R (min)	K (min)	α Angle (deg)	MA (mm)
Control	0.6 ± 0.2	1.1 ± 0.2	76.0 ± 0.5	66.7 ± 4.5
GNS/Alg-NGs-270	26.7 ± 0.6	N/A	1.2 ± 0.2	2.1 ± 0.3
Warfarin	0.9 ± 0.3	1.5 ± 0.4	70.1 ± 0.7	59.8 ± 3.1
Rivaroxaban	2.3 ± 0.3	3.3 ± 0.5	50.9 ± 0.8	44.6 ± 2.9
Dabigatran	*30.0*	N/A	N/A	N/A
Enoxaparin	*30.0*	N/A	N/A	N/A

(B)



Inhibitor	R (min)	K (min)	α Angle (deg)	MA (mm)
Control	4.1 ± 0.3	0.9 ± 0.2	76.3 ± 1.5	64.5 ± 3.5
GNS/Alg-NGs-270	13.8 ± 0.3	4.2 ± 0.1	42.6 ± 1.2	N/A
Warfarin	7.6 ± 0.4	1.2 ± 0.3	70.3 ± 1.8	65.8 ± 3.1
Rivaroxaban	*30.0*	N/A	N/A	N/A
Dabigatran	10.3 ± 1.3	1.8 ± 0.4	67.0 ± 1.6	59.2 ± 2.9
Enoxaparin	*30.0*	N/A	N/A	N/A

Fig. S20 Comparison of the anticoagulation effects of GNS/Alg-NGs-270 and commercial drugs at a concentration of 0.25 mg mL^{-1} on (A) thrombin-activated and (B) kaolin-activated TEG. The TEG measurement for human blood in the absence of anticoagulant material was used as a control. N/A: not available. Interim values are surrounded by asterisks*.

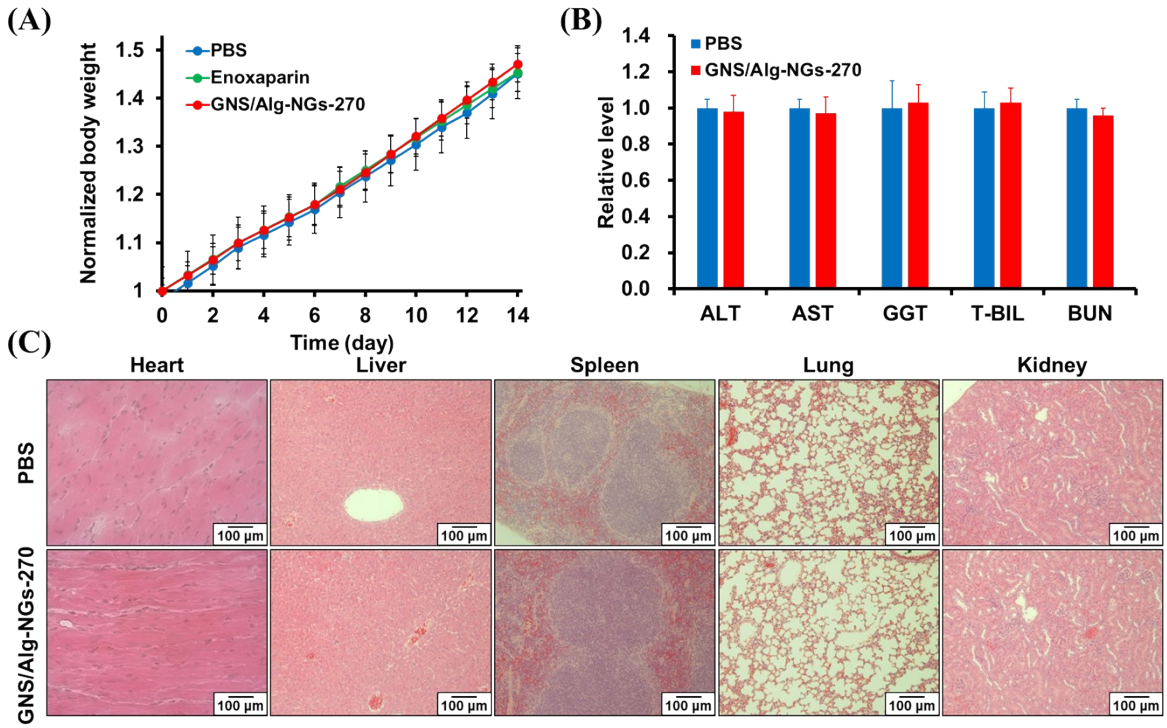


Fig. S21 (A) The normalized body weight of male Sprague-Dawley rats for 14 days, (B) relative levels of ALT, AST, GGT, T-BIL, and BUN in the blood, and (C) H&E staining of the tissues excised of male Sprague-Dawley rats at day 15 after the intravenous (IV) injection with PBS, enoxaparin, and GNS/Alg-NGs-270. Body weight 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 male Sprague-Dawley rats on Day n and Day 0, respectively). Error bars in (A) and (B) represent the standard deviations of experiments consisting of measurements for twenty and five rats, respectively.

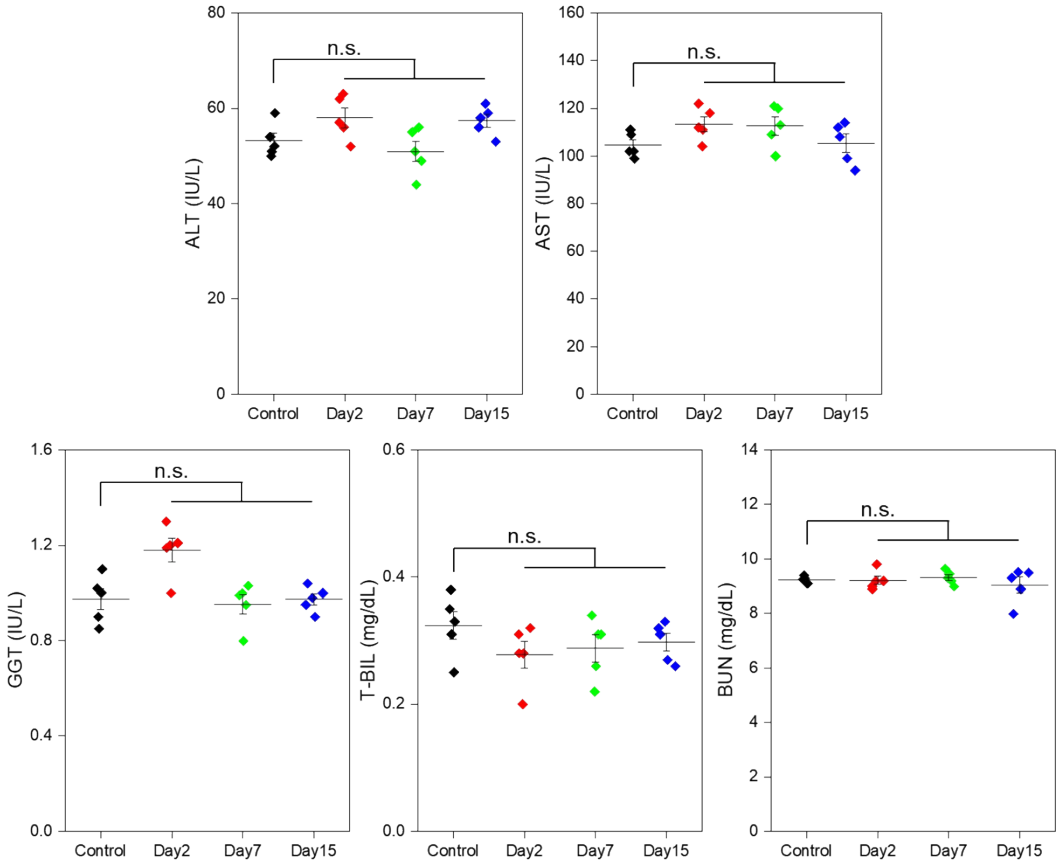


Fig. S22 ALT, AST, GGT, T-BIL, and BUN levels in the blood after the intravenous injection of GNS/Alg-NGs-270 (1 mg kg^{-1}) for 2, 7, and 15 days from each group (n = 5). n.s. indicates no significant difference.